A STUDY OF PHOSPHOFRUCTOKINASE ACTIVITY
IN ERYTHROCYTES

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ABSTRACT

Human blood was collected and stored in a standard acid-citrate-dextrose medium (ACD) and acid-citrate medium (AC) at 5°C. Phosphofructokinase (PFK) activity was measured in hemolysates prepared from these bloods by coupling the enzyme activity with diphosphopyridine nucleotide reduction and measuring the optical density changes at 340 mμ in a Beckman quartz spectrophotometer.

In all hemolysates of bloods stored in ACD, there was a decrease from the initial PFK activity of 70-80% by the 25th day and 80-90% by the 35th day. A similar decrease was observed in the bloods stored in AC but the changes occurred earlier. PFK became rate-limiting in the catabolism of fructose-6-phosphate at the time glycolysis fails in stored erythrocytes.

The high initial level of PFK activity in blood was prolonged by the addition of adenosine to the ACD and AC media. For 35 days AC plus adenosine was superior to ACD as a PFK preservative. The addition of adenosine to blood during storage resulted in a regeneration of PFK activity. Experiments with hemolysates, prepared from blood stored in ACD, preserved in adenosine, inosine, ribose-5-phosphate, and adenosinetriphosphate suggest a pathway of adenosine metabolism in the erythrocyte. The mechanism of the protective action of adenosine on PFK in the erythrocyte is considered from the above findings.

Y.S. BROWNSTONE.

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SECTION I

INTRODUCTION

Two enzyme systems in living tissues are responsible for the degradation of carbohydrate for energy purposes, glycolysis and the tricarboxylic acid cycle. Glycolysis is the enzymatic energy - producing mechanism within the erythrocyte. It is known to fail after blood has been stored for two weeks under conventional conditions.

Recently, Blanchaer (1, 2) and Pappuis et al (3) have postulated that the site of this failure lies somewhere in the glycolytic scheme whereby glucose is transformed into fructose diphosphate. A study of phosphofructokinase (PFK) has therefore been undertaken in an attempt to locate this site and deals with the following problems:

(1) The development of a spectrophotometric assay method for measuring PFK activity.

(2) Observations on the rate of PFK activity in hemolysates of fresh and stored red cells.

(3) A comparison of PFK activity and the known glycolytic behaviour of stored blood.

(4) The possibility of regenerating PFK activity by introducing additives to the preservative media.

(5) Investigations into the pathway of utilization of such an additive.

SECTION II

GLOSSARY OF TERMS AND ABREVIATIONS

AC - acid-citrate medium.

ACD - acid-citrate-dextrose medium.

ACA - acid-citrate-adenosine medium.

ACDA - acid-citrate-dextrose-adenosine medium.

aden. - adenosine.

ADP - adenosinediphosphate.

ATP - adenosinetriphosphate.

"......-blood" - blood preserved in the medium indicated.

DPN - diphosphopyridine nucleotide.

DPN.H_2 - reduced diphosphopyridine nucleotide.

F-6-P - fructose-6-phosphate.

FDP - fructose-1,6-phosphate.

G-3-P - glyceraldehyde-3-phosphate.

GPD - glyceraldehyde-3-phosphate dehydrogenase.

Indigenous enzymes, indigenous enzyme activity- refers collectively to the erythrocyte enzymes:aldolase, triosephosphate isomerase, and GPD.

inos. - inosine.

PFK - phosphofructokinase.

PGK - phosphoglycerate kinase.

Preserved, stored - these terms are used interchangeably.

R-5-P - ribose-5-phosphate.

Ru-5-P - Ribulose-5-phosphate.

TI - triosephosphate isomerase.

SECTION III

REVIEW OF THE LITERATURE

The purpose of this section is to provide a brief background to the subject material and is divided as follows:

METHODS OF STUDY OF ERYTHROCYTE ACTIVITY

Investigation into the properties of the erythrocyte by previous workers has followed two general methods:

(A) Study of the intact red cell in its own plasma or in another medium.

(B) Study of hemolysates prepared from the isolated red cells.

Each of these methods has its own advantages and limitations, Method A being generally employed in studying over-all cell activities and Method B for studying specific reactions.

Method A has been used by Rapaport (7), Engelhardt and Ljubimova (27) and Parpart (8, 28) in their studies on the rate of glycolysis in stored red cells as measured by the rate of disappearance of glucose from stored blood and the rate of lactic acid formation, the passage of ions in and out of the red cell, the osmotic and dimensional changes in erythrocytes during storage and the viability of stored cells.

Method B, the study of hemolysates, has been employed by Dische (30, 31, 32) to study the enzymic breakdown of adenosine by the erythrocyte. Blanchaer (1,2) has employed this method to measure

the rates of activity of various glycolytic enzymes. The hypothesis presented is that the hemolysate reflects quantitatively the activities of the intact red cell. The main advantage of this method is the ability to isolate and study individual cellular enzyme reactions and to introduce any desired intermediate of red cell activity or other material that may otherwise be excluded from the reactive cell content by the semi-permeable cell membrane.

GLYCOLYSIS AND VIABILITY OF THE ERYTHROCYTE

Investigations into the biochemical nature of the erythrocyte have increased during the past two generations as a result of the problem arising out of the preservation of whole blood for transfusion during the two world wars. The red blood cell has a life span of approximately 120 days in the human body (4). There is a loss in viability of erythrocytes preserved in the standard acid-citrate-dextrose medium (ACD) at 5°C. as has been shown by the post-transfusion survival of these cells (5,6). Associated with this loss is the failure of the red cells' ability to utilize glucose in the production of energy (3,7,8,9).

Glycolysis has been observed in blood since Pavy noticed in 1854 that glucose disappeared in shed blood and the blood became acid (10). Lepine introduced a term glycolysis in 1890 (10). More recently it has been considered that glycolysis is the major system by which the red cell produces energy to maintain itself (10, 11, 12, 13). Enzymes similar to those known to make up the glycolytic

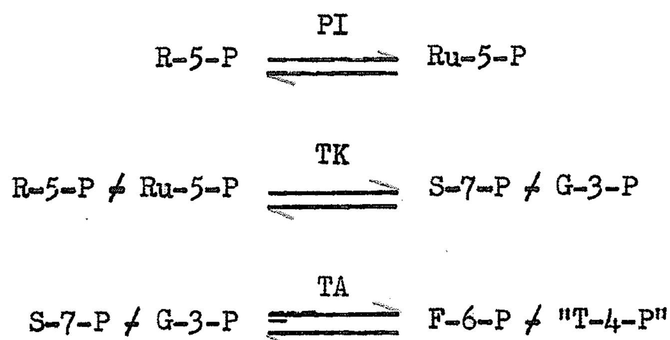
scheme in muscle (11,12) have been exhibited in the erythrocyte (fig. 1) in studies of the intact cell (11,12) and hemolysates (2). This energy mechanism maintains itself at a normal rate for two weeks in stored blood, but by the third week there is an impairment in its ability to catabolize glucose (3).

Investigation into the failure of stored erythrocytes to maintain glycolysis has shown that there is an increase in accumulated pyruvate in stored erythrocytes (14,3) and a progressive decrease in the ability of these cells to reduce added pyruvate (1). However, it has been shown that the enzymes disposing of trioses in hemolysates prepared from stored red cells persist at a high level activity for more than three weeks (1,2). The suggestion has therefore been made (1,2,3) that the site of glycolytic failure in stored erythrocytes occurs in the steps leading to the transformation of glucose to fructose-1,6-diphosphate (fig. 1,A). This study was undertaken to investigate the activities of one of the enzymes in these steps, phosphofructokinase (PFK), in fresh and stored red blood cells.

ASSOCIATED CARBOHYDRATE ENZYME SYSTEMS IN THE ERYTHROCYTE

Two other carbohydrate enzyme systems associated with glycolysis (fig. 1,A,B, and C) have been described (34,35,36,37) and have been found to be present in the erythrocyte (30,32,38). A brief description of these enzyme systems (33,34,40,41) is given below with special emphasis on the catabolism of pentose by these pathways:

<u>The pentose cycle</u> (fig. 1,C): Starting with ribose-5-phosphate (R-5-P), an equilibrium mixture of R-5-P and ribulose-5-phosphate (Ru-5-P) is formed by the action of an enzyme, pentose isomerase (PI). This mixture of R-5-P and Ru-5-P is transformed by the enzyme transketolase (TK) to sedoheptulose-7-phosphate (S-7-P) and glyceraldehyde-3-phosphate (G-3-P). The formation of fructose-6-phosphate (F-6-P) and a tetrose phosphate ("T-4-P") is a result of the activity of the enzyme transaldolase (TA) on S-7-P and G-3-P. These reactions, respectively, are described below:



F-6-P is then catabolized by way of the glycolytic scheme (fig. 1,A). The net result, energetically, is the build-up of high energy bonds in the form of ATP.

<u>The Hexosemonophosphate Shunt</u> (fig. 1,B): Glucose-6-phosphate can be directly oxidized enzymatically to 6-phosphogluconic acid (6 PG) by a triphosphopyridine nucleotide specific dehydrogenase. 6 PG is then decarboxylated enzymatically to Ru-5-P and carbon dioxide. An equilibrium mixture of Ru-5-P and R-5-P is formed by pentose isomerase and the catabolism of pentose from this point follows that

of the pentose cycle described above.

The early work of Dische (30) with hemolysates of erythrocytes was a pioneer step in the recognition of two enzymic systems related to glycolysis in the degradation of carbohydrate in living tissues. He found that adenosine added to hemolysates of red blood cells resulted in esterification of inorganic phosphate and the disappearance of the ribose of the nucleotide. R-5-P, triose phosphate, FDP and a glycolaldehyde phosphate accummulated. Dickens (33) working with a crude yeast extract postulated the existence of a scheme for the stepwise oxidation of glucose that is now known as the Hexosemonophosphate Shunt (fig. 1). The presence of this shunt in other living tissues has been reported (41,43,44) and recently a study of oxidative glycolysis in human erythrocytes has been reported by Dubovsky and Sonka (42). The existence of an additional cycle, called the "Pentose-Cycle"by Horecker (34), has been recognized in a variety of living tissues: erythrocytes (32), liver (36), and yeast (40). The postulated system (fig. 1,C) confirms the views of Dische that ribose, split from the nucleotide, is phosphorylated, broken down into smaller fragments, and then resynthesized to hexose esters.

STUDIES OF THE CHARACTERISTICS OF PFK IN PLANT AND ANIMAL TISSUES

PFK (fig. 1) is the enzyme responsible for the formation of fructose-1,6-diphosphate (FDP) from fructose-6-phosphate (F-6-P).

Its activity has been observed in plant and animal tissues and the purified enzyme has been extracted from these materials. Meyerhof (15) noted in yeast and muscle that adenosinetriphosphate (ATP) was required for the formation of FDP. By measuring the phosphate transferred and isolating the FDP formed, Ostern et al (16) showed that muscle extracts catalyze this reaction. Dische (17) observed the presence of PFK in red blood cells. Engelhardt and Sakov(18) showed that PFK was highly sensitive to oxidizing agents. Meyerhof and Wilson (19) noted a 20-40% decrease in PFK activity on the addition of octyl alcohol and its homologues to a brain preparation of PFK. Working with PFK prepared from rabbit muscle, Racker (20) developed a method for the spectrophotometric measurement of PFK activity in a series of reactions leading to the breakdown of F-6-P to 3-phosphoglycerate (3 PG) and the accumulation of reduced diphosphopyridine nucleotide ($DPN.H_2$). He also proposed a method of PFK measurement using the series of reactions of F-6-P to FDP to dihydroxyacetone phosphate (DHAP) to alpha glycerophosphate with the concurrent oxidation of reduced DPN ($DPN.H_2$) to oxidized DPN. The importance of ATP as a substrate to PFK in addition to F-6-P has been reported in that no FDP is formed from F-6-P in the absence of ATP in yeast and animal tissue extracts (21,22). Taylor (23,24) prepared an aqueous extract of PFK from rabbit muscle with the enzyme content equal to 1% of the extractable protein. Using a test system similar to that of Racker (20) he found that PFK was inactivated upon dialysis and

that it required bivalent magnesium ions (Mg^{++}) for its activity. Meyerhof and Geliazkana (29) found that they could maintain failing glycolysis in brain tissue homogenates by repeated addition of ATP. Muntz (25) prepared PFK from brain tissue. He pointed out the necessity of the presence of an excess of both substrates, F-6-P and ATP, for optimum PFK activity. Decreasing either substrate from the optimum resulted in a decrease of activity. Using the extraction method of Taylor (23), Ling and Hardy (26) have succeeded in isolating a PFK preparation more active than that of Taylor from rabbit muscle. Inosinetriphosphate (ITP) and uridinetriphosphate (UTP) could replace ATP as substrate.

STUDIES WITH ADENOSINE AND ITS EFFECT ON THE ERYTHROCYTE

Dische (30), working with hemolysates, early demonstrated that adenosine is utilized by living tissue, first by a splitting off of the pentose and then by the phosphorylation, degradation, and reformation of the pentose to hexose phosphate. Later (32) he postulated the existence of two closely related cycles in the erythrocyte capable of degrading glucose to CO_2 and of catabolizing pentoses. In more recent studies, Prankerd and Altman (58,59) have found that adenosine forms a substrate for the regeneration of the essential energy stores of the red cell and that adenosine may substitute for glucose in mediating the phosphate exchange in the red cell. Gabrio *et al* (39,60,61) have shown that incubating stored

blood with adenosine resulted in regeneration of organic phosphates, improvement in osmotic fragility, an increase in intracellular K^+, and an improvement in post-transfusion viability as compared with the control "ACD-blood".

ENZYME-SUBSTRATE RELATIONSHIPS

A consideration of certain aspects of substrate-enzyme relationships is necessary to aid in the explanation of the effect on PFK activity of adenosine addition to stored blood.

Substrate has been defined as the material upon which enzymes act (45). However substrate may act not only as the base material for enzyme activity, but it may also protect the specific enzyme against destructive forces in the reaction medium. The classical experiment, reported by Berger, Slein, Colowick and Cori (46), best illustrates this point. They found that they could achieve a four-fold purification of crude yeast hexokinase by letting a concentrated crude solution stand in the presence of its substrate, glucose. In experiments where glucose was not used, hexokinase was destroyed by proteolytic enzymes. These conclusions are confirmed by McDonald (47) in a description of hexokinase preparation which states that the protective action of glucose prevents the inactivation of hexokinase by trypsin.

SECTION IV

METHODS

The general plan of experimentation was to collect series of bloods, subject these bloods to predetermined conditions of storage, and to apply various tests to samples of these bloods. A detailed description of the handling of the blood, the tests applied and the preparation of all materials employed follows. Table I describes the preservative media used in the various series and the additives introduced to stored blood.

PREPARATION OF PRESERVATIVE MEDIA

Preparation of the Acid-Citrate-Dextrose Medium

Acid-citrate-dextrose medium (ACD) was prepared according to the specification of the Canadian Red Cross Society (48) as follows:

50 grams dextrose

41.6 grams disodium acid citrate

These were dissolved in double distilled water (DDW) and made up to a volume of 2000 milliliters. The ratio of whole blood to be collected to preservative medium was calculated to be four in one and the required volume of preservative was transferred into the collection flask.

Preparation of the Adenosinetriphosphate Medium

Preparation of the bacterial filter: ATP, introduced to preserved hemolysate, could not be sterilized in the autoclave

because of the lability of its terminal hydrogen phosphate bonds. Therefore, aliquots of ATP were sterilized by running them through a bacterial filter.

The filter candle was prepared by the following methods used by the Department of Bacteriology, Faculty of Medicine, University of Manitoba (49). The candle was sterilized in the steam autoclave at 20 pounds, 130°C., 20 minutes and then dried. It was placed in a muffle furnace and heated until it became glowing red (at approximately 900°C.) and then cooled. Three volumes of DDW were passed through the filter in each direction and then the whole apparatus was assembled. The apparatus was sterilized in the autoclave and dried. It was then ready for use.

The efficiency of the viral filter was checked by passing through a bacteria in broth suspension. After filtration this broth was incubated for 72 hours at 37°C. and tested at intervals for signs of bacterial growth by the Bacteriologist, St. Boniface Hospital. The tests were negative.

Filtration through the bacterial filter: The material to be sterilized was dissolved in a minimum volume of DDW and passed through the filter. The filter was washed with succeeding small volumes of DDW. The mouth of the collection tube was flamed and sealed with a sterile cap. Volumes of solution were extracted with sterile needle and syringe and transferred to sterile bottles for use in the hemolysate preparation.

PREPARATION OF BLOOD FOR TESTING

Collection of Blood for Preservation

Blood was collected in such a manner as to avoid clotting, hemolysis, bacterial and chemical contamination. All materials and apparatus used were previously sterilized in a steam autoclave at 15 pounds pressure, 110°C. for 20 minutes.

These included 16 gauge needles, a graduated flask containing the preservative medium (1/4 the volume of blood to be collected), rubber and metal cap to the collection flask, suction trap and all its connections, and rubber hosing.

Sixteen gauge needles were used for taking venous blood. When the flow of blood from the donor to the collection flask was slow, a slight suction was applied to the flask to shorten the collection time and thus avoid clotting. The collection flask was gently swirled throughout the collection period so that the blood would be admixed with the preservative medium and clotting was thus prevented. Collection time was 4 - 5 minutes. The rubber cap was swabbed with cotton soaked with 70% alcohol and covered with a metal screw cap. The flask was then plunged into an ice-water bath to a height equal to the level of blood in the flask. When the temperature of the flask and its contents had fallen to that of the bath, it was placed in a refrigerator maintained at 5 ± 1°C. and stored.

Another portion of the fresh blood was collected directly

into a tube containing heparin and a sample of this was immediately tested for PFK activity.

Transfer of Blood from the Collection Flask into Preservative Bottles

In series B and C, where blood was preserved in media other than ACD, the method of collection was similar to that described above. The blood was first collected in a graduated flask and then predetermined amounts were transferred into smaller bottles containing the desired preservative variation. It was postulated that by collecting a single sample of blood in one container and then subdividing this a more accurate comparison between the various preservative media could be made than if the blood from the donor was collected directly into the bottles containing the various media. This method assured the introduction of aliquots of a uniform sample of blood into the various preservative bottles and avoided the possibility of variation in composition in successive portions of blood from the same donor.

A stock acid-citrate (AC) solution of the required concentration was prepared. A volume one-half that normally required for preservation (or 1/8 the volume of blood to be collected) was transferred into the collection flask. This volume was sufficient to prevent clotting. Into the preservative bottles were placed 1/8 the volume of the same AC solution that contained, in addition, an amount of the preservative additives that would equal the desired

concentration when the bottles were filled with blood to the desired volume. When the blood was transferred into the preservative bottles, the predetermined final concentration of preservative plus additive was attained.

In all the bloods tested in series B, 40 ml. of the stock AC solution was transferred into the collection flask. For the ACD preservative 25.0 mg. of oven-dried dextrose (cooled in dessicator) were transferred into the preservative bottle and were dissolved in AC solution to a final volume of 10 ml. For the ACA preservative, a similar procedure as the preceeding was used with 285.0 mg. of adenosine being dissolved in the AC solution. For the ACDA preservative, 25.0 mg. of dried dextrose and 285.0 mg. of adenosine was dissolved in the AC solution. The resultant blood-preservative mixture, upon the transfer of AC blood to each of these bottles, would have a final volume of 100 ml. containing 80 ml. whole blood in 20 ml. preservative.

In other blood, series B, where dl-glyceraldehyde was used instead of adenosine, and in series C, where various concentrations of adenosine were added, the procedure was similar to the above.

The preservative bottles containing the various media were tightly capped with porous paper and loosely covered with aluminum foil. These were sterilized in a steam autoclave at 15 lbs. pressure, at 110°C. for 20 minutes. When they were removed a tight seal was made with the aluminum foil. Tight fitting rubber caps

for these bottles were also sterilized.

The methods of taking the blood and collecting it in the collecting flask were as described above. The procedure used in transferring the blood from the flask to preservative bottles will now be set forth: (all materials mentioned, except the blood, were previously sterilized in a steam autoclave at 15 lbs., 110°C., for 20 minutes.)

After the blood was taken the flask was inverted several times and gently shaken to ensure a thorough mixing of the contents. The cap was swabbed with a cotton ball soaked in 70% alcohol. An air bleeder that reached to the bottom of the flask was introduced through the rubber cap. A 13 gauge needle, attached by an adapter to a rubber tube with a bell-bottom drain at the other end, was introduced through the cap. The flask was inverted and attached to a vertical stand. The flow of blood was controlled by releasing a clamp on the rubber tubing. The paper and foil covering were removed from the preservative bottle. The bottle was placed under the collection flask, the bell-bottom drain placed over it and the "AC-blood" was allowed to run in to a predetermined 100 ml. level. During the transfer the bottle was gently swirled to ensure a mixing of the contents. The bottle was immediately capped, inverted several times, and placed in an ice-water bath. Transfer of "AC-blood" to the other three bottles was completed in successive operations as the above. The bottles were then stored in a

refrigerator operated at 5°C. Employing two people, both the taking and transferring of blood were completed in approximately 15 minutes after the start of the blood flow from the donor. This method proved to be efficient from the point of view of sterile technique and the absence of hemolysis and clotting.

Sampling

A method of taking a sample of blood from the preservative bottles for PFK assay and planned to avoid the possibilities of contamination of blood, hemolysis or activation of its glycolytic mechanism is described below. After the first sample had been taken, i.e. after the rubber cap had been pierced with a needle, a piece of cotton soaked in 70% alcohol was placed on the cap and sealed with parafilm covering.

The bottle was removed from the refrigerator and placed in an ice-water bath of a volume that was greater than the height of blood in the bottle and below the level of the cap. The bottle was inverted and gently shaken until a homogeneous mixture was obtained within it. The parafilm and cotton were removed and the cap was swabbed with cotton soaked in 70% alcohol. A sample of blood was gently removed from the inverted bottle using a sterile 21 gauge needle and sterile syringe. The bottle was placed into the ice-water bath, covered with a piece of cotton soaked in 70% alcohol, and sealed with a parafilm covering and placed in the refrigerator at 5°C. Using this method, contamination was avoided in bottles sampled many times over a period of as long as 90 days; hemolysis

as a result of handling was at a minimum, and glycolysis in the cells was retarded by providing an environment of 5°C. at all times.

Addition of Adenosine to Blood During Storage

In the methods described below for adding adenosine to blood during storage, a control portion of the same blood was always kept without adding adenosine to it for use in comparing the effects of the added adenosine.

The desired weight of adenosine was transferred into a bottle in a minimum of DDW, sterilized and the bottle sealed as above. A predetermined volume of blood mixed with ACD was aseptically transferred from preservative bottle using a syringe and needle into this bottle. The contents were well mixed and the bottle stored at 5°C.

Preparation of Hemolysate for Storage

The hemolysate was prepared in such a manner as to avoid the possibilities of contamination and deterioration of the sample. Blood was collected into ACD and transferred into a 50 ml. constricted-neck centrifuge tube (as described above - Methods II,2.). The blood was centrifuged at 1000 X gravity for 10 minutes in the refrigerator (5°C.). The supernatant plasma, ACD solution and surface layer of red cells were aseptically withdrawn with the aid of suction. A volume of sterile DDW equal to that of the packed red cells was introduced. The contents of the bottle were well mixed, alternately frozen and thawed three times to effect complete hemolysis and then

placed in the refrigerator at 5°C. for storage.

Preservative materials were introduced in a similar manner as described above (Methods II, 4.). The desired volume of hemolysate was aseptically transferred to a sterile bottle containing the material dissolved in a minimum of DDW. The reliability of the aseptic method employed was illustrated in the unsuccessful attempt to culture any bacterial growth from samples of hemolysate, prepared in the described manner, in nutritive broth and plates incubated 72 hours at 37°C.

PHOSPHOFRUCTOKINASE ASSAY

PFK was measured in hemolysates prepared from preserved red cells, using the reactions shown in Figure 2. In devising the method, it was assumed that the properties of erythrocyte PFK were similar to those of the muscle enzyme (20,24,26). In the assay system conditions were so arranged that PFK was the rate-limiting enzyme in the series of reactions leading to the reduction of DPN, which was detected by an increase in optical density at 340 mµ in a Beckman DU quartz spectrophotometer. F-6-P and ATP were the substrates in this system. The cofactor of PFK, magnesium, was added to ensure optimum activity of the enzyme. The erythrocyte has been reported to be deficient in aldolase (2) and therefore crystalline aldolase was added to assure the removal of FDP as rapidly as it was formed. Potassium fluoride was added to inhibit the enzyme enolase (11) thus assuring the accumulation of $DPN.H_2$ by preventing its removal in the reduction of pyruvate to lactate.

<u>Method</u>

The preservative bottle was carefully shaken and the parafilm with the cotton were removed. The rubber cap was swabbed with a cotton ball soaked in 70% alcohol. The sample of preserved blood was aseptically removed with a sterile needle and syringe (see SAMPLING, this section) and suspended in a centrifuge tube containing an ice-cold 1.15% KCl solution. The cells were washed three times in the centrifuge at 5°C. with 1.15% KCl solution. To make the hemolysate, 0.2 ml. of a 50% washed cell suspension in potassium chloride solution was frozen and thawed three times. Ice-cold DDW was added to 14 ml. and the stroma removed by centrifugation at 1000X gravity for fifteen minutes at 5°C.

The materials used in the assay are shown in Table 2 indicating the order that they were added to a set of matched 1 cm. light-path cuvettes. The blank cuvette contained all materials except the added DPN. The final volume was 3.35 ml. and volume changes due to additions during the assay were corrected to this figure. The final pH was 8.5. The cell housing of the spectrophotometer was maintained at a temperature of 36 $\pm$ 0.5°C. by pumping water through it from a constant temperature bath.

After the addition of each of the last five materials (Table 2) the change in optical density at 340 mμ was read at one minute intervals in the spectrophotometer against the blank. Temperature equilibration was complete before ATP was added to initiate the PFK reaction.

The readings were plotted on a graph comparing the time elapsed with the changes in optical density. Appropriate corrections were made for changes in density due to dilution resulting from the addition of materials to the cuvettes. Corrections were also made for variations in the concentration of cell material present by comparing the hemoglobin concentrations of the hemolysates (see Results). The hemoglobin concentration in the hemolysates was determined as follows:

The method used was an adaptation of the cyanhematin method of King (50). To one ml. of hemolysate was added 4 ml. of 0.125N HCl. This solution was allowed to stand ten minutes, and then 1 ml. of 6% NaCN added. This solution was transferred to 1 cm. light-path cuvettes and the optical density, using a DDW blank, was read at 540 mμ, slit width 0.02 mm., on the Beckman DU quartz spectrophotometer. A typical value of hemoglobin concentration of 13.2 grams % in the hemolysate was chosen as a standard. In each of the hemolysates tested, the slope of PFK activity was corrected by comparing its hemoglobin concentration with that of the typical value.

PREPARATION OF ENZYMES AND SUBSTRATES

Preparation of Aldolase and GPD

Crystalline aldolase was prepared from minced rabbit muscle according to the method of Taylor (51) and GPD according to the method of Cori (52). These purified materials were lyophilized at - 75°C. and stored *in vacuo* at - 20°C. The enzymes remained

stable for more than two years when preserved in this state.

The aldolase preparation was tested for PFK and GPD contamination using the substrate-enzyme system described in Table 3, I and II. The purity of GPD was determined by testing for PFK and aldolase contamination using assay systems I and II (Table 3). The general principle followed in these tests was to provide substrates for possible contaminants of the enzyme and otherwise complete the system leading to the reduction of DPN (Fig. 2). Contamination of GPD and aldolase with PFK or with each other would lead to an increase in optical density at 340 mμ.

In the test systems, readings were taken after the addition of DPN and after the addition each of the materials following DPN in the order listed in Table 3. In test system I, the absence of PFK contamination in both the enzymes tested was shown by the absence of DPN reduction following the addition of F-6-P and GPD. Aldolase was shown to be responsible for initiating DPN reduction by the addition of FDP (Fig. 9, I). In test system II aldolase was shown to be free of GPD contamination by the absence of DPN reduction in a system containing DPN, F-6-P, FDP and aldolase. GPD addition resulted in a prompt increase in optical density (Fig. 9, II). In test system III GPD was shown to be free of aldolase activity in a system containing DPN, F-6-P, FDP, and GPD (Fig. 9, III).

Preparation of Substrates

The substrates FDP, F-6-P, the substrate ATP and the additive R-5-P were originally bought in the form of the barium salt. To avoid introducing ions into the assay system that do not normally occur in the red cells, the barium ions were exchanged for potassium ions. This was performed using Dowex-50 cation exchange resin. This resin has a milli-equivalent exchange value of 4.86 per gram dry weight (53). Six grams of resin were transferred into an ion exchange column. The resin was washed with DDW and 1N HCl was passed through the column to remove adsorbed ions and saturate the column with hydrogen ions. The column was washed with DDW until the excess (non-adsorbed) hydrogen ions were removed. This point was considered to be reached when the filtrate was neutral to pH indicator paper. The column was then saturated with potassium ions by passing through a solution of 1M KCl. The excess ions were removed by washing with DDW. The column was now considered ready for proceeding with the process of exchanging the barium ions for potassium ions in the desired material. The substrate salt was dissolved in a minimum volume of 0.1N HCl and passed through the column. The column was then washed with successive volumes of DDW to ensure removal of all the substrate and until the required volume of filtrate was achieved. The solution was then tested for the absence of barium.

Crystalline sodium pyruvate was prepared according to the method of Lardy (64) from pyruvic acid. A pure white powder was

obtained after recrystallizing three times.

Preparations of Other Assay Materials

Phosphate buffer: The preparation of 0.1M phosphate buffer was a modification of the method described by Umbreit (54). Solutions of 0.1M K_2HPO_4 and 0.1M KH_2PO_4 were mixed to a pH of 7.4 (measured electrometrically).

Cysteine: Cysteine was prepared daily before the assay was begun.

Adjustments in pH

In all cases where pH adjustments were made electrometrically in solutions to be used in the assay, KOH and HCl solutions were used for this purpose.

PHYSICAL AND CHEMICAL TESTS APPLIED TO THE ERYTHROCYTE

It has been shown (7,8) that some physical tests applied to preserved blood can be used as an index in estimating the condition (i.e. viability) of these cells. A number of physical tests were therefore applied to blood stored in various media to compare the changes in PFK activity with physical changes in the cells. Chemical tests were made to follow the variation in the concentration of various cell constituents.

Mechanical Fragility Tests

The mechanical fragility tests were performed by Mr. J.M. Beaton as a physiology project in his second year in the Faculty of Medicine at the University of Manitoba. The method followed was

that described by Shen, Castle and Fleming (55).

Osmotic Fragility Tests

The osmotic fragility tests were carried out by Mr. T.S. Anderson as a physiology project in his second year in the Faculty of Medicine at the University of Manitoba. The method followed was that outlined by Shen, Ham and Fleming (56).

Mean Corpuscular Hemoblobin Concentration Determinations

The mean corpuscular hemoglobin concentration (M.C.H.C.) has been shown to be a reliable measurement of the changes in red cell size during storage (7). It was calculated from the hemoglobin concentration (Hb) and hematocrit (Hcrit.) of whole blood samples as follows:

$$\text{M.C.H.C.} = \frac{\text{Hb grams \%}}{\text{Hcrit. \%}} \times 100$$

Hematocrit Determinations: The hematocrit was measured using a Wintrobe, constant bore, graduated tube. This was filled with a sample of blood to a minimum volume of 75% of the tube's capacity. The tube was centrifuged for 30 minutes at 3,000 r.p.m. All samples were tested in duplicate. The deviation of results between duplicate samples was not more than $\pm$ 0.5%.

The hematocrit was calculated as follows:

$$\text{Hematocrit (as \%)} = \frac{\text{Volume of packed cells}}{\text{Total volume of sample}} \times 100$$

Hemoglobin Concentration Tests: Hemoglobin concentration of the whole blood was measured according to the cyanhematin method

of King (50).

Total Adenine Determinations

The total adenine concentration in whole blood and plasma was measured spectrophotometrically according to the method described by Albaum *et al*(57).

SECTION V

RESULTS AND DISCUSSION

The results of this study will be presented under the topical headings related to the four series of bloods tested. As presented in detail below, these deal with the PFK assay in fresh and stored blood and the factors affecting it, the effects of additives on PFK activity in stored blood, and the tests of PFK activity in stored hemolysates.

THE PHOSPHOFRUCTOKINASE ASSAY AND THE FACTORS THAT EFFECT THE ASSAY

PFK Assay

PFK was measured in hemolysates prepared from fresh and preserved red cells, using the series of reactions shown in Figure 2. In the assay system the conditions were so arranged that PFK was the rate limiting enzyme in the sequence of reactions (Fig. 2) leading to the reduction of DPN. This was detected by an increase in optical density at 340 mμ (62). Series of tests were carried out to establish the validity of the method. The results will be presented below in the section "Results of Testing the Method."

The typical assay for PFK activity is shown in Figure 3. In this example and in all the hemolysates studied there was a prompt linear increase in density upon the addition of ATP (Fig. 3, slope B) due to the reduction of DPN by D-glyceraldehyde-3-phosphate formed from F-6-P and ATP by the reaction shown in Fig. 2. With each hemolysate tested, the rate of DPN reduction was estimated from

the slope of the section of the assay curve corresponding to curve B in Fig. 3. Enzyme activity was expressed in micromoles DPN reduced per gram hemoglobin per minute using a molar extinction co-efficient of 6.3×10^3 (65).

In all the specimens of "ACD-blood" tested there was a decline in PFK activity during storage at 5°C. as illustrated in Figures 10, 11, 12, and 13. The decline in all these bloods was approximately 50-60% from the inital value by the 15th day of storage; 70-80% by the 25th day; 80-90% by the 35th day after the beginning of storage as compared with the fresh blood sample tested from the same donor. In these samples, studied at later date (Fig. 11), PFK activity was only 10% of the original activity on the 45th day and less that 1% on the 80th day after storage.

Indigenous Enzyme Activity

It has been reported that the erythrocyte level of aldolase activity is low when compared with the high levels of GPD and PGK (2). Therefore in the ordinary assay the hemolysate system was fortified with crystalline aldolase (51) to assure the removal of FDP as rapidly as it was formed by the activity of PFK. In a separate assay, the indigenous level of activity of red cell enzymes below PFK in the glycolytic scheme (aldolase, TI, and GPD) after periods of storage (series B,C, and D, Table 1), was examined by omitting crystalline aldolase in the assay system and FDP was the substrate added. A typical assay is plotted in Figure 3, curve A. As is shown in A, Figures 10, 11, and 12, the level of activity in 8 bloods

tested in series A,B, and C, remains fairly constant during storage. Sections A and B of the same figures illustrate comparisons between the levels of PFK activity and the levels of indigenous enzyme activity below PFK in the same hemolysates prepared from fresh and stored blood. These illustrations show that PFK activity falls below that of the indigenous enzymes in stored erythrocytes after 14-18 days of storage. This is the time that glycolysis has been reported to fail in stored erythrocytes (3).

Results of Testing the Method

In devising the present assay, it was assumed that the PFK enzyme in the blood has similar properties to that of the muscle enzyme (20). To establish the validity of the assay method it was necessary:(1) to show that PFK was in fact the rate-limiting enzyme in this system; (2) to show that $DPN.H_2$ accumulation was actually being measured by the changes in optical density in the spectrophotometer; (3) to demonstrate the necessity of each of the materials used in the assay system excluding those of the buffer and DDW; (4) to test the effects of hemolysate preparation on PFK activity; (5) to exhibit the reproducibility of the results. These are considered in detail below:

Was PFK rate limiting? If it could be shown that the glycolytic enzymes in the hemolysates below PFK (aldolase, TI, and GPD, Fig. 2: II, III, and IV respectively) could dispose of an added excess of FDP, the product of PFK activity, at a faster rate than

it was being formed, then it could be said that PFK activity was rate-limiting in this series of reactions. FDP (1.4 mM.) was added to the assay system after PFK activity had been initiated approximately 25 minutes earlier. There was a prompt linear increase in the rate of DPN reduction as reflected in the changes of optical density at 340 mμ (slope C, Figs. 3,5,6, and 8).

The increase in the rate of DPN reduction indicated that the enzymes in the test system below PFK are capable of handling FDP at 5-10 times the rate it is produced by the hemolysate PFK activity. This finding was confirmed in all the hemolysates tested (slope C in Figs. 10,11, and 12). Since the slope after FDP addition to the assay was relatively constant over a period of 0-12 weeks in all hemolysates it was considered that the enzyme systems below PFK remain relatively intact after prolonged storage. Further evidence to illustrate this point is presented below.

Doubling the F-6-P added to the assay system produced no further increase after DPN reduction had been initiated for some time (Fig. 4, curve 2, slope B).

The above tests show that the slope of the linear density increase following ATP addition (slope B, Fig. 3) was indeed a measure of PFK activity, since neither concentration of substrate, nor the subsequent reaction disposing of the product of FDP was rate-limiting in the reduction of DPN.

In other tests, to show that PFK was the limiting factor

in this series of reactions, duplicate assays were performed in which a control pair of cuvettes contained the assay materials and the test pair contained all assay materials except F-6-P. The control and test assay are shown in Figures 4, curves 1 and 3 respectively. There was no reduction of DPN in the test cuvettes (Fig. 4, curve 3, slope B) until FDP was added to them (Fig. 4, curve 3, slope C). This indicates that PFK is the limiting factor in this series of reactions and no reaction occurs if its substrate is not present.

Was the optical density change a measure of DPN reduction? The spectrophotometric assay method for measuring PFK activity (described above) by arranging conditions so that PFK was the rate-limiting enzyme leading to the reduction of DPN by GPD was similar to the methods of Racker (20) and Ling (26). To show conclusively that the accumulation of DPN.H_2 was being measured, sodium pyruvate was added to the cuvettes at the end of the assay. There was an immediate drop in the optical density (Figs. 4,5,6,7, and 8) caused by the rapid re-oxidation of DPN.H_2 in the reduction of pyruvate to lactate by lactic acid dehydrogenase activity (Fig. 1,A) to approximately the same value as at the beginning of the assay. This immediate response to pyruvate addition was observed in all samples of hemolysates tested either fresh, or up to 80 days storage.

In some of the assays, the changes in optical density were followed at 410 mμ. The stable values observed at this wave length confirm the conclusion that the changes observed at 340 mμ

were specific to DPN-DPN.H_2 transformations since both compounds have the same extinction co-efficient at 410 mμ, their isobestic point.

Were the added materials necessary to the assay system?

(i) Arsenate: Tests were carried out to compare a system with and without added arsenate. The omission of arsenate resulted in a decreased rate of DPN reduction (Fig. 5, curve 3, slope B) as compared with the duplicate sample containing the usual assay materials (Fig. 5, curve 1, slope B). Other tests showed that the addition of phosphate in the concentration used in the assay system, as a buffer, was found not to interfere with the stimulatory effect of arsenate (Fig. 5, curve 2, slope B) by comparing assay systems with (Fig. 5, 1) and without (Fig. 5, 2) added phosphate.

(ii) Cysteine: Krimsky (66) has reported that glutathione and cysteine are activators of muscle GPD and that cysteine addition resulted in a 20-fold increase in enzyme activity as compared with no added cysteine. To ensure the rapid removal of glyceraldehyde-3-phosphate (G-3-P), cysteine has been included in the assay system. The effect of cysteine and glutathione was compared in duplicate samples of hemolysate by including cysteine in one and an equimolar concentration of glutathione in the other. The inclusion of cysteine resulted in a more rapid reduction of DPN (Fig. 7, 1) as compared to the system where glutathione replaced it (Fig. 7, 2). When cysteine was added to the cuvettes 20 minutes after glutathione

the result was a prompt increase in the rate of DPN reduction (Fig. 7, 2).

(iii) Magnesium: Magnesium ions have been reported as co-factors for PFK (20). Omission of magnesium ions in the assay system resulted in no reduction of DPN.

Did the method of hemolysate preparation introduce a bias into the experiments? The possibility that the method of hemolysate preparation (vis à vis the washing of the cells) might introduce a bias into the experiments was considered. It was thought that washing the cells might result in the selection of the hardier cells by causing the osmotic and mechanical hemolysis of the ones whose cell wall were weaker. To discover whether such was the case, the following test was done on various samples of blood, both fresh and after various periods of storage. One half of each of the samples was washed in the manner previously described (see Methods) and the other half was immediately centrifuged, the supernatant liquid removed by suction, and the hemolysate was prepared in the usual manner. The results (Table 5) showed that there was no difference in PFK activity in the cells washed or unwashed, whether in fresh blood or blood stored for 28 days.

Were the results reproducible? The ability to reproduce results, one of the measures of reliability of an experiment, was investigated between individual samples of the same bloods and within each series of bloods where a variation was introduced. Two

forms of reproducibility were examined:

Analytical reproducibility: This can be defined as the effectiveness of the analytical method to reproduce results, within experimental error, in duplicate samples.

Biological reproducibility: This may be defined as the characteristic of specific biological reactions to be reproduced in living samples of similar tissue from different donors.

At intervals, two separate samples of the same blood were consecutively withdrawn from the same bottle of blood, treated in the typical manner for assay, and assayed for PFK activity. The results were two parallel slopes with identical values for the rate of DPN reduction (Table 6). This showed that the method was analytically reproducible. The reproducibility of results between various bloods within the various test series has been reported, in part, above and will be presented below in this section. The results of the various bloods tested within each series were similar (Figs. 10,11,12, and 13), indicating that the activity studied was biologically reproducible.

(i) Hemoglobin concentration: To further standardize all assay results, corrections were made for slight variations in the number of red cells used for hemolysate preparation. Hemoglobin concentration was considered to be the best method of estimating the amount of red cell material present in the hemolysate since it was a direct measurement of the number of cells used. The slopes of

the rate of DPN reduction were corrected in each sample to a typical hemoglobin of 13.2 gm. %.

To test the reliability of this procedure, assays were run on a number of hemolysates using various dilutions of the same hemolysate to see if the rate of PFK activity was indeed proportional to the hemoglobin content. A typical test is shown in Figure 6. The dilutions of the hemolysate used for the PFK assay that exhibited the activity of curves 1, 2, and 3 had an optical density of 0.264, 0.198 and 0.132 respectively when measured in the spectrophotometer for hemoglobin concentration. The results, illustrated in Figure 6, slopes B, show that the hemoglobin content accurately reflects the amount of cell material present in the hemolysate. On the other hand, variation in the hemoglobin content of hemolysates prepared at various dates during storage bore no relationship to the changes in PFK activity.

Discussion

The study carried out on hemolysates showed that the erythrocyte PFK enzyme was similar in properties to that of the muscle enzyme. PFK in the blood converted F-6-P and ATP to FDP and ADP. The absence of either of the substrates resulted in no change in the optical density in the assay medium.

Since glycolysis continues at a constant rate during the first 2 weeks of storage in ACD (3) in spite of the sharp drop in PFK activity noted here, it is unlikely that PFK limits the over-all

rate of erythrocyte glycolysis during this interval. Supporting this conclusion is the observation that the removal of FDP was a much slower process than its formation by PFK (Fig. 10,11,12) during the first 16 days. Thereafter PFK activity fell below that of the enzymic reactions utilizing FDP, indicating that PFK became rate-limiting at about the time that glycolysis usually begins to fail in preserved erythrocytes.

THE EFFECT OF ADDITIVES TO PRESERVED BLOOD ON PFK ACTIVITY

With the establishment of the nature of PFK activity in fresh and stored blood, attempts were made to prolong the period of high activity of this enzyme exhibited in fresh blood. Two additives to the blood preservative medium were used: dl-glyceraldehyde and adenosine.

The Effect of Glyceraldehyde Additive on PFK Activity in Preserved Blood

The suggestion has been made that the failure in glycolysis in preserved blood occurs somewhere above the triose stage (1,2,3,). It was thought that glyceraldehyde might circumvent this block or failure and provide substrate material for glycolysis. PFK could then be stimulated by the reformation of the triose into hexoses, thus providing substrate for this enzyme. In these tests (Table 1) there was no beneficial effect observed. It was observed

that PFK activity was lower in blood preserved with glyceraldehyde as compared to the same blood in ACD only. In addition the cells showed less resistance to osmotic hemolysis and greater mechanical fragility (Table 4) and there was spontaneous hemolysis visible in the preservative bottles after 14 days storage.

The Effect of Adenosine Added to the Preservative Medium on PFK Activity in Preserved Blood

Introduction: Various effects have been ascribed to the addition of adenosine to the preservative medium on the activities of the erythrocytes (38,39,59). Adenosine was added to the preservative media to discover whether PFK activity was similarly affected. A number of physical tests (osmotic and mechanical fragility, cell size) were made on the same samples assayed for PFK activity to compare the metabolic changes exhibited by PFK activity with the physical changes.

A preliminary test was applied to blood that had been stored four weeks. An aliquot of "ACD-blood" was aseptically withdrawn and transferred to a bottle containing sterile adenosine solution (20 mM/L) and stored for another week. The presence of adenosine in the "ACD-blood" resulted in a regeneration of PFK activity to 187% (Fig. 8, 1) of that of the same "ACD-blood" (Fig. 8, 2). Preliminary tests with three concentrations of adenosine (5 mM/L, 10 mM/L and 20 mM/L) showed no adverse osmotic effect on erythrocyte structure microscopically.

The series of tests that followed (series B and C, Table 1) diverged along a number of lines. Adenosine was added to the preservative medium at the beginning of storage and blood was collected into media containing: a) ACD, b) AC, c) ACA (5 mM/L aden.), d) ACDA (5 mM/L aden.). Each of the preceding samples acted as controls for the ones that followed in the order described. Various concentrations of adenosine in the preservative media were compared (5,10, and 20 mM/L). Adenosine was added at various days after the beginning of storage to all the bloods being tested. A comparison was made among the various samples of the same blood by running simultaneous PFK assays on various dates of storage. The effect of adenosine on the level of activity of the indigenous enzymes was also measured in a number of bloods.

The addition of adenosine to the preservative medium or to stored blood resulted in an increased PFK activity in all the hemolysates tested as compared to the control sample of the same blood to which no adenosine was added. The detailed results follow.

Series B (Figs. 11,14, and 15): In those samples of series B where fresh blood was added to a preservative medium containing adenosine (10 mM/L), PFK activity decreased slowly as compared to the ACD and AC control bloods. PFK activity in "ACA-bloods" remained above that of "ACD-bloods" for approximately six weeks and in "ACDA-blood" for eight weeks (Figs. 14 and 15). The

general pattern exhibited by the PFK activity in these "adenosine-bloods" was a sustained high level period of activity for the first two weeks, a gradual decline in the following three weeks and a sharp decrease thereafter.

On the 7th day this was reflected in an activity of 150% of the control (150%/C) "ACD-blood" or a 5% drop from the activity of fresh blood (5%/I). On the 20th day, when "ACD-bloods" were 40%/I and below the level of indigenous enzyme activity, the "adenosine-bloods" exhibited an activity 200%/C or 75-80%/I. On the 35th day, when "ACD-bloods" were less than 20%/I, "ACA-bloods" were equal to them and "ACDA-bloods" exhibited a value of 200%/C or 40-50%/I and were approximately equal to the level of indigenous enzyme activity. The results in series C-2 (Fig. 17, "ACDA-10") where 10 mM/L adenosine was in the ACDA preservative medium, were similar to those in series B, confirming this general pattern.

Series C (Figs. 12,16, and 17): 5 mM/L of adenosine was added to AC and ACD preservative in series C and the differentiation in PFK activity, as compared with the AC and ACD controls, was not as distinct. However the activity in the "adenosine-bloods" remained higher than the ACD and AC controls for a period, as in series B, but then dropped to the same level. As can be seen from these graphs (Figs. 16 and 17, "ACDA-5"), the curves of activity in the adenosine bloods over a period of seven weeks, are almost parallel to those of the ACD controls at a slightly higher level and fall to the same

level by that time.

In series C, where 20 mM/L of adenosine was introduced initially in the preservative medium, a variation to the above pattern appeared (Fig. 17, "ACDA-20"). There was an initial increase in PFK activity above the activity in fresh blood. On the 20th day this equalled 135% of the activity of one day old "ACD-blood" taken at the same time and remained above this level for approximately four weeks. The level dropped to 50% by six weeks but remained above the indigenous enzyme level for seven weeks and still maintained a level of 30% at the end of ten weeks storage.

In series C, an attempt was made to follow the passage of total adenine nucleotides from the plasma to the red cell by calculating the red cell content from measurements of the plasma and whole blood contents (57). Only a qualitative indication was gathered from these tests that there was an increase in red cell nucleotides in the adenosine bloods. The method used appears to lack specificity in that too many other substances absorb this wave length.

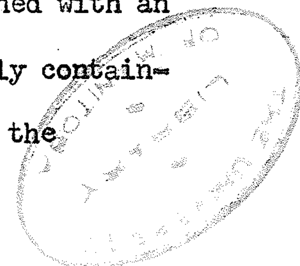
In the tests with various concentrations of adenosine in the preservative medium (ACD, ACD + 5 mM/L, ACD + 10 mM/L, ACD + 20 mM/L, AC + 5 mM/L, and AC + 10 mM/L) the effects were similar (Figs. 14, 15, 16, and 17). The degree of regeneration of erythrocyte PFK activity in these bloods and the duration of this effect varied directly with the concentration of adenosine present. The effects were transient and by the 45th day all the bloods but that

containing ACD ≠ 20 mM/L adenosine had reached the "ACD-blood" level of PFK activity.

The effect of added adenosine on certain physical characteristics of erythrocytes: Concurrent physical tests were carried out on the same samples of blood (series B, 4,5, and 6) to see if adenosine exerted a protective action on the erythrocytes as reported in the literature (60,61). *In vitro* tests have shown that osmotic fragility (56), mechanical fragility (55), and cell size of the erythrocyte (7), are known to reflect the viability of stored erythrocytes (7) and its ability to maintain glycolysis (7,8). A definite protective action was exhibited by the "adenosine-bloods". As shown on Table 4, osmotic fragility, mechanical fragility and the increase in cell size were less in the "adenosine-bloods" as compared to the other bloods tested after 28 days of storage. The results of the tests on the effects of adenosine on cell size were confirmed in series C-2.

The Effect of Adenosine Added to Preserved Blood During Storage on PFK Activity

The addition of adenosine to stored blood at various dates after the beginning of storage resulted in a prompt regeneration of PFK activity. In series C-1, (Fig. 16, "ACD-≠ adenosine") 5 mM/L adenosine was added to "ACD-blood" on the 22nd day of storage. There was an increase of 200%/C in the PFK activity. Similar results (Fig. 16, "ACDA ≠ adenosine") were obtained with an equimolar addition of adenosine to "ACDA-blood" originally containing 5 mM/L adenosine. Four weeks following the addition the



activity had fallen to the same level as the original "ACD-blood". A further addition of adenosine to these bloods on the 40th day resulted in an increase similar in magnitude to the previous increase and was followed by a rapid decline in activity (Fig. 16, "ACD + adenosine" and "ACDA + adenosine").

When 5 mM/L adenosine were added to preserved blood originally containing 10 mM/L added adenosine, results were similar to the above (Fig. 17, "10 + adenosine"). However, on the 23rd day of storage, when 5 mM/L of adenosine was added to "ACDA-blood" containing 20 mM/L adenosine added on 0 day, the result was a prolongation of the high level of activity in the blood (Fig. 17, "20 + adenosine"). It remained above the first day level for more than six weeks following storage and did not fall below the indigenous enzyme level till ten weeks later.

The level of indigenous enzyme activity in the bloods to which adenosine was added was unaffected by this addition (Fig. 8, curve 3, slope A) and remained constant for ten weeks at the same level as the "ACD-blood".

To investigate the possibility that adenosine was having a direct stimulatory affect on PFK activity in the hemolysate assay system, adenosine of equimolar concentration present in hemolysates prepared from 20 mM/L "ACDA-blood" was added to the assay system after activity had been initiated for some time by the addition of ATP. There was no effect on the rate of DPN

reduction and the slope of activity was unaltered. It was concluded that no direct effect was being exerted by adenosine in the assay system.

Discussion

The effect of adenosine added to "ACD- and AC-bloods" may result from:

a) A direct protective action of adenosine on PFK by some unknown mechanism.

b) A substrate protective action of adenosine on PFK.

A direct protective action of adenosine on PFK activity: A preliminary test, whereby adenosine was added to the PFK assay medium, showed that there was no direct effect of adenosine on PFK activity. However, the possibility that adenosine may have some direct protective effect on PFK in the erythrocyte has not yet been excluded by these experiments.

A substrate protective action of adenosine on PFK activity: The effect of adenosine added to "ACD- and AC- bloods" on PFK activity appears to be of a substrate nature. The transitory effect that could be regenerated on further addition of adenosine and the similarity in the slopes of progressively decreasing PFK activity in "ACD-blood" and the "adenosine-bloods" support this conclusion. The observation that adenosine had no effect on the enzymes responsible for the removal of FDP lends further support to the possibility that adenosine added to stored

blood protected PFK activity by providing ATP and/or F-6-P as substrate.

PFK is known to be a labile enzyme (see Review of Literature) inactivated by a variety of conditions including a low pH medium and various oxidizing agents. If substrate is provided by adenosine catabolized in the erythrocyte, it could protect the enzyme against such inactivation.

The effect of adenosine on PFK activity and on some physical characteristics of the erythrocytes reported here was similar to its effects on a number of factors in stored erythrocytes reported by other workers, including: rate of glycolysis, post-transfusion survival, osmotic and mechanical fragilities, and cell size. Rubinstein _et al_ (38) have shown that the rate of glycolysis, as measured by the increased accumulation of lactate, was increased by the addition of adenosine to ACD. They showed, as did Prankerd and Altman (58,59), that there was a regeneration of organic phosphates and ATP in the red cell upon the addition of adenosine to the preservative medium. Gabrio and co-workers (39,60) have demonstrated that the "storage lesions" (increased osmotic and mechanical fragilities, increased cell size, depletion of organic phosphates, and decreased post-transfusion survival) of stored erythrocytes were reversed upon the addition of adenosine to the preservative medium. Successive additions of adenosine to the preserved blood prolonged the duration of these effects. These

authors suggest that the effect of adenosine is by way of a build-up of the essential energy stores in the erythrocyte. They suggest (38,39), as did Dische (32), that adenosine is deaminated, the ribose phosphorytically cleaved, and that the pentose phosphate catabolized to hexose and triose phosphates resulting in a net gain of ATP. An investigation into the pathway of adenosine utilization as related to PFK activity is presented below.

EXPERIMENTS WITH PRESERVED HEMOLYSATES

Introduction

When the effects of adenosine on PFK activity added to stored blood had been observed, the problem of locating the pathway of utilization of adenosine was explored. Recently various authors have described the existence of a pentose cycle in various living tissues: red cell (38), yeast (34), liver (36), and bacteria (41). Adenosine is metabolized by way of this cycle to such glycolytic intermediates as F-6-P and triose phosphate (Fig. 1). It was postulated therefore that adenosine might be metabolized by way of a similar series of reactions by red cell enzymes of a similar nature to those of this pentose cycle (Fig. 1,C). The net gain to the glycolytic scheme would be approximately 2 moles of F-6-P for every 3 moles of adenosine metabolized and a resultant gain in energy of approximately 4 moles of high-energy phosphate bonds as ATP (36,41). If such a cycle does exist in the red cell,

then the intermediate products of adenosine catabolism, inosine, R-5-P and ATP should exhibit a similar effect on PFK activity as adenosine when added to the red cell. However, the red cell is impermeable to R-5-P and ATP. It was decided therefore to store hemolysates aseptically prepared from "ACD-blood" and observe the results of adding these materials to hemolysates.

<u>Results: Series D (Figs. 13, 18, 19 and 20)</u>

Hemolysate was prepared from "ACD-blood" (see Methods) and stored at 5°C. The hemolysate PFK activity (Fig. 13, II) was compared with that of the "ACD-blood" (Fig. 13, I) from which it had been prepared over a period of 3 weeks. The slope of PFK activity over the storage period was similar in both cases, although the activity in the hemolysate decreased more rapidly after the first few days (Figs. 13, I and 13, II) perhaps because of the disappearance of substrate. Adenosine was added to hemolysate prepared from "ACD-blood" and the effect on PFK activity (Figs. 18, I, "D-2 ≠ aden." and 18, II,"D-3 ≠ aden.") was similar to that observed in "ACD-blood" (Fig. 16, "ACD ≠ adenosine"). There was a transient regeneration of PFK activity. By three weeks after the addition, PFK had fallen to 20% of the regenerated value.

With the observation that the hemolysate PFK enzyme activity curve closely resembled that of "ACD-blood" with and without added adenosine (Fig. 18), this system was accepted as reliable for comparing the affects of added R-5-P and inosine with added

adenosine to hemolysates. The addition of ATP was included in the series as well to see if the build-up of ATP was the resultant major effect of the adenosine (28,61) utilized by the cells.

Hemolysates prepared from "ACD-blood" were aseptically transferred into five bottles. One sample was the control hemolysate. Each of the other four bottles contained one of the following substances that would equal a final concentration of 20 mM/L when the hemolysate was added to it: adenosine, R-5-P, inosine and ATP.

In a preliminary test added adenosine and R-5-P were compared. Both had similar effects on PFK (Fig. 19 I, "D-4 + aden." and "D-4 + R-5-P"). The additions resulted in a prolongation of the high level of PFK activity in the hemolysates for 20 days and then decreased rapidly to the level in the control hemolysate by the 40th day.

Adenosine, R-5-P, and inosine had similar effects upon the PFK activity of the other hemolysates tested (Fig. 19, II "D-5 + aden.", and "D-5 + R-5-P, and "D-5 + inos."). There was a regeneration of PFK activity to a minimum of 200% of the control hemolysate. The activity in each specific case decreased slowly for a week and fell off rapidly for the following ten days. The "ATP-hemolysate" (Fig. 19, II, "D-5 + ATP") showed an initial rise in PFK, but this fell off rapidly during the first week to a level equal to that of the control hemolysate and there was no change in the ten days that followed.

Discussion

Since the products of adenosine catabolism, inosine, R-5-P, and ATP, were found to have similar effects on PFK activity in hemolysates as does adenosine itself, it is likely that a pentose cycle mechanism exists in the erythrocyte. This finding supports the observations of Dische (32) and more recently of Rubinstein *et al* (38).

The protective action on PFK exhibited in the experiments with hemolysates, as well as with the intact cells, appears to arise out of the production of more ATP as a high energy substrate for PFK activity. This conclusion is supported by the observation that adenosine, inosine, and R-5-P all show a similar initial rise in PFK when added to hemolysates, as does ATP and an increased ATP production results from their catabolism. The more prolonged effect of adenosine, inosine and R-5-P as compared with ATP may arise from the following sources:

a) It may take time for the build-up of additional ATP from the catabolism of these substances *via* the pentose cycle and glycolysis and this could result in a continued supply of ATP until the substrates disappear. ATP added directly is immediately available as substrate for PFK and may thus disappear earlier.

b) A specific adenosinetriphosphatase, known to be present in the erythrocyte, may be active in the preserved hemolysate. The action of this enzyme may cause the breakdown of the added ATP

and may result in the rapid removal of this substrate.

c) As previously noted, the catabolism of 3 moles of pentose can result in the formation of 4 moles of high energy phosphate bonds as ATP. Comparing the results, therefore, of an equimolar addition of ATP and any of the above pentose-containing substances could be erroneous in that more substrate as ATP is actually being formed as a result of the catabolism of the latter.

d) The formation of F-6-P from R-5-P may have some additional protective action on PFK activity to that of ATP by some unknown mechanism.

SECTION VI

SUMMARY OF RESULTS

Introduction

This study of phosphofructokinase activity in erythrocytes was undertaken in attempt to locate the site of glycolytic failure (7,10) in stored blood. Previous research (2,3) established the possibility of locating this site in the steps leading to the breakdown of glucose to FDP in the glycolytic scheme. The possibility of PFK being this site was therefore investigated. The objectives were as follows:

1. To develop a reliable spectrophotometric assay procedure for measuring PFK activity in hemolysate of whole blood.

2. To study PFK activity during prolonged periods of storage in "ACD-blood" and compare its characteristics with the known glycolytic behavior of stored blood.

3. To attempt to prolong PFK activity with additives to the preservative medium and correlate this with effects on other cell characteristics.

4. To identify the action of substances that promoted the prolongation or regeneration of PFK activity in stored blood.

The plan of the experiment was organized into four series of experiments patterned after the four objectives listed above. In all series a portion of each normal human venous blood was collected into an ACD bottle. This portion of the blood collected was used as the control sample throughout all the series of

experiments.

Series A was conducted to establish the reliability of the PFK assay and to establish the behavior of PFK in the red cells during storage.

Series B was carried out to attempt to prolong the initial high level of PFK activity observed in fresh blood. Beside "ACD-blood" additional blood was transferred into bottles containing ACD plus 133 mM/L dl-glyceraldehyde, AC plus 66.5 mM/L glyceraldehyde and 16.5 mM/L dextrose, ACD plus 5 mM/L adenosine, ACD ≁ 10 mM/L adenosine, AC ≁ 10 mM/L adenosine and AC. Concurrent tests on the physical changes in the cells were conducted on the change in osmotic fragility, mechanical fragility, and cell size of the stored blood.

Series C was undertaken to examine the nature of the effect adenosine had on the PFK activity of stored blood, whether it was temporary or prolonged, whether various concentrations of adenosine had differing effects. In addition to maintaining "ACD-blood" as control, blood was collected in AC, AC plus 5 mM/L adenosine, ACD plus 5, 10, and 20 mM/L adenosine. Also, aliquots of each of these bloods were subdivided at various dates during storage and were added to bottles containing 5 mM/L adenosine. The indigenous enzyme levels were measured as well as PFK activity. To follow the passage of added adenosine, total adenine nucleotides were measured in the whole blood and plasma. The red cell content was

calculated from these figures and the hematocrit of the sample being tested.

Series D was carried out to discover the pathway of adenosine utilization by the red cell. Hemolysates were prepared from ACD controls stored at 5°C. and these were considered controls in the experiments that followed. To individual samples of these hemolysates were added one of each of the following: adenosine, 20 mM/L; R-5-P, 20 mM/L; inosine, 20 mM/L; ATP, 20 mM/L. These hemolysates were stored at 5°C. and then PFK activity was measured during storage.

The summary of results will be presented in the following order:

1. Reliability of the PFK Assay
2. Decreased PFK Activity in Preserved Blood.
3. Indigenous Enzyme Activity in Stored Blood.
4. The Effect of Added Glyceraldehyde on PFK Activity in Preserved Blood.
5. The Effect of Added Adenosine on PFK Activity in Stored Blood.
6. The Pathway of the Adenosine Effect on PFK Activity—Experiments with Stored Hemolysate.

Reliability of the PFK Assay

The spectrophotometric method of assaying PFK activity in hemolysates prepared from stored blood has been examined from a number of points of view, and has been shown to be reliable by the

following results:

a) Under the conditions of the assay it has been shown that PFK is the rate limiting factor in the metabolism of F-6-P to 3PG and therefore in the reduction of DPN. Without the addition of substrate F-6-P no PFK activity is observed. It has been shown that neither the concentration of substrate, nor the subsequent reactions disposing of the product FDP, were rate limiting.

b) The accumulation of DPN.H_2 has been shown to be the substance measured by the spectrophotometer in the assay.

c) When duplicate samples of hemolysate prepared at the same time from the same bloods were assayed for PFK activity, the results varied by less than $\pm$ 2%. This figure is well within the experimental limits of error of $\pm$ 5%. The pattern of changes in PFK activity in ACD stored blood was similar in all blood tested.

The functions of the following materials have been demonstrated:

a) Arsenate has been shown to increase the rate of DPN reduction in this system. This effect is attributed to the simplification of the glycolysis of G-3-P to 3PG.

b) Cysteine promotes the rapid reduction of DPN in the system. Accumulation of the latter substance is almost non-apparent in the spectrophotometer when the former is ommitted from the assay system. The effect is attributed to the requirement

of --SH groups by the enzyme GPD and to the inactivity of this enzyme in its absence.

c) The system required added magnesium ions, F-6-P and ATP to function. Magnesium is a cofactor to PFK. ATP and F-6-P are substrates for PFK activity and ATP was used to initiate DPN reduction in this assay system.

d) The low indigenous level of aldolase in erythrocytes was found to be a limiting factor in the assay when PFK activity was high during the first two weeks of storage. Crystalline aldolase was added to the assay system to assure the prompt removal of FDP formed by the action of PFK activity.

Hemoglobin concentration is a reliable measure of the concentration of cellular material (i.e. enzyme material) in the hemolysate prepared from the erythrocyte. In hemolysates prepared from the same blood sample the rate of PFK activity varied directly with the hemoglobin content.

Decreased PFK Activity in Preserved Blood

Fifteen separate samples of blood were collected into ACD preservative solution and tested over a period of 0-12 weeks for PFK activity. In all there was a similar pattern of decreased PFK activity during storage. The fresh blood exhibited a high level of activity. In fifteen days the activity dropped 50-60%, 70-80% in 25 days, 80-90% by day 35 and 90-100% in 90 days.

In series B and C, where AC as well as ACD was the preservative medium, the decrease in PFK activity followed a similar pattern as the above, but the changes occurred earlier.

The significance of these results, indicating that the decreased PFK activity in preserved blood becomes an important factor in the glycolytic failure of blood stored at 5°C in ACD after the 16th day of storage, has been discussed.

Indigenous Enzyme Activity in Stored Blood

The activity of the indigenous enzymes, aldolase, triose isomerase and glyceraldehyde-3-phosphate dehydrogenase were measured collectively in a hemolysate system without added aldolase and using fructose-1, 6-phosphate as substrate in four different bloods (Series A, B and C). The level of activity was unchanged in the fresh bloods tested and up to the 76th day after storage. Adenosine added to "ACD-blood" or to "AC-blood" had no effect on the activity of these enzymes and the level was the same as in "ACD-blood". Aldolase, TI, and GPD activity persisted at the same level in AC preserved blood.

The Effect of Glyceraldehyde on PFK Activity in Preserved Blood

The addition of glyceraldehyde to the preservative medium (66.5 mM/L and 133 mM/L) adversely affected blood during storage. There was a decrease in PFK activity as compared to "ACD-blood" prepared on the same dates from one donor. The erythrocytes were

less resistant to osmotic and mechanical fragility tests and increased in size more rapidly than the ACD control. Spontaneous hemolysis was visible in the preservative bottles containing glyceraldehyde after two weeks of storage.

The Effect of Adenosine on PFK Activity in Stored Blood

The addition of adenosine to the preservative medium resulted in an increased PFK activity in the hemolysates. When adenosine was included in ACD at the beginning of the storage period, the decrease in PFK activity was slower and activity remained at a higher level for a longer time, as compared with "ACD-blood". The protective effect of adenosine was approximately proportional to its concentration in the preserved blood. In the experiments where adenosine to a final concentration of 5 mM/L was added to the preservative medium at the beginning of storage (Series C), the PFK level in the erythrocytes of the preserved bloods as compared with that of the "ACD-blood" control on the same days was 100-110% on the 1st day, 110-150% on the 7th day, 110-140% on the 21st day and equal on the 35th day. Starting with 10 mM/L adenosine final concentration (Series B and C) the PFK activity in ACDA as compared to its ACD control was 100-200% on the 7th day, 120-200% on the 20th day, 200% on the 35th day (equal to the level of the indigenous enzymes), equal by the 50th day. With 20 mM/L adenosine at the beginning of storage the fresh

blood level of activity was maintained for three weeks and the activity remained above that of the indigenous enzymes for seven weeks. The PFK activities in the "ACDA-blood" as compared with its "ACD-blood" control were 105% on the 1st day, 230% on the 8th day, 350% on the 20th day, 275% on the 37th day, decreasing slowly from that day to the 75th day, the last day tested.

Adenosine added to AC in the preservative medium (Series B and C) resulted in similar effects to the PFK activity of blood preserved in this medium as in the "ACDA-bloods". The effects were not as prolonged but, in the concentrations tested (5 mM/L and 10 mM/L), ACA proved to be a better PFK preservative medium than ACD, for at least 35 days.

Concurrent physical tests on some of these bloods (Series B) indicated that adenosine has a protective action on the red cell resistance to mechanical and osmotic fragility during storage, as well as protecting it against swelling. The fragility of the cells in the adenosine preservatives was one-half those in the ACD and the degree of swelling was less than in the "ACD-blood" tested on the same day.

The addition of 5 mM/L adenosine to "ACD-blood" during storage (5 mM/L) resulted in a regeneration of PFK activity of approximately 200% when added on 15, 20, 25, or 40 days after storage. The same results were obtained when adenosine was added to blood already containing 5 and 10 mM/L of adenosine. Initially

there was a rapid decrease in activity parallel to the decreased activity observed in control "ACD-blood" and, by twenty days after addition, the level of PFK activity dropped to approximately that of the ACD control. In the test where adenosine was added to blood preserved for three weeks in ACDA containing 20 mM/L of adenosine, the effect was to prolong the existing high level of PFK activity. The decrease in activity was slow and dropped to that of the 20 mM/L "adenosine-blood" approximately fifty days after the addition. PFK activity remained above that of the indigenous enzyme level for ten weeks.

The significance of these results, indicating that adenosine added to preserved blood exhibits a substrate like effect on PFK activity, has been discussed.

The Pathway of the Adenosine Effect on PFK Activity-Experiments with Stored Hemolysate

Hemolysates were prepared so that the effect of the probable intermediate products of adenosine catabolism, unable to enter the red cell, on erythrocyte PFK activity could be tested. PFK activity in hemolysates prepared from "ACD-blood" exhibited a similar slope to time (days) comparison curve as the "ACD-blood" only that it persisted for a shorter period of time at similar levels. Adenosine (20 mM/L) added to the hemolysates resulted in a regeneration in PFK activity similar to that of adenosine added to "ACD-blood", followed by a decrease to the original level. The

addition of equimolar amounts of R-5-P and inosine result in similar, almost identical effects on the PFK activity of aliquots of the same hemolysate prepared from "ACD-blood". Added ATP (20 mM/L) resulted in a regeneration of PFK activity approximately equal to that of added adenosine. However, the effect persisted for only half the time of the adenosine effect.

The significance of these results, indicating the existence of a pentose cycle and its effects on PFK activity, has been discussed.

SECTION VII

CONCLUSIONS

1. The glycolytic enzyme phosphofructokinase that is present in the erythrocyte is similar to that found in muscle tissue. It is inactive in the absence of its substrates, ATP and F-6-P, or its cofactor, magnesium. The rate of PFK activity in erythrocytes has been measured in a system similar to that described for this enzyme in muscle tissue.
2. Phosphofructokinase activity in the erythrocyte decreases progressively during storage of whole blood in ACD at 5°C. By the 25th day the enzyme has lost 70-80% of initial activity in fresh cells. PFK has proven to be an important factor in the slowing of glycolysis in erythrocytes after 16-18 days of storage of "ACD-blood". The rate at which it converts F-6-P to FDP, at that time, becomes progressively slower than the rate of FDP removal by the enzymes below it in the glycolytic scheme.
3. The level of activity of the indigenous enzymes, aldolase, triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase collectively, remains constant in erythrocytes stored for 80 days.
4. Glyceraldehyde added to preserved blood has adverse effects on PFK activity and physical characteristics of the erythrocyte.
5. The high initial level of PFK activity in blood is prolonged by the addition of adenosine to the preservative medium. For 35

days AC plus adenosine was a superior PFK preservative to ACD. The addition of adenosine to blood during storage results in regeneration of PFK activity.

6. Experiments with stored hemolysates indicate the existence of a pentose cycle-mechanism in erythrocytes for the catabolism of adenosine. These experiments suggest that the protective effect of adenosine is a result of the formation of ATP as substrate.

SECTION VIII

TABLES AND FIGURES

TABLE 1

PLAN OF THE STUDY

The Series of Bloods Tested, The Preservative Media and Additives Used, The Division and Subdivision of the Blood and the Time After the Beginning of Storage of these Divisions

Series A

Blood Number	Portions (preservative media, conc.)
1	ACD*
2	ACD
3	ACD
4	ACD
5	ACD

Series B

Blood Number	Portions (preservative media, conc.)	Divisions (additive, conc., day)
1	A. ACD	a. A b. A+aden. (20mM/L, 26th day)
2	A. ACD B. AC+D(13.6mM/L)+dl-glyceraldehyde(66.5mM/L) C. AC+dl-glyceraldehyde(133mM/L)	
3	A. ACDA(5mM/L aden.)	
4	A. ACD B. ACDA(10mM/L aden.) C. ACDA(20mM/L aden.)	
5	A. ACD B. AC C. ACDA(10mM/L aden.) D. ACA(10mM/L aden.)	
6	A. ACD B. AC C. ACDA(10mM/L aden.) D. ACA(10mM/L aden.)	

* ACD : 15.4mM/L AC and 27.2mM/L D(48) .

TABLE 1 (cont.)

Series C

Blood Number	Portions (Preservative, conc.)	Division of Portions (additive, conc., day)	Subdivision (additive, conc., day)
1	A. ACD	a. A	
		b. A+aden.(5mM/L,22nd day)	(i) b
			(ii) b+aden.(5mM/L,40th day)
	B. ACDA(5mM/L aden.)	a. B	
		b. B+aden.(5mM/L,22nd day)	(i) b
			(ii) b+aden.(5mM/L,40th day)
	C. AC	a. C	
		b. C+aden.(5mM/L,22nd day)	
	D. ACA(5mM/L aden.)	a. D	
		b. D+aden.(5mM/L,22nd day)	
2	A. ACD		
	B. ACDA(5mM/L aden.)	a. B	
		b. B+aden.(5mM/L,23rd day)	
	C. ACDA(10mM/L aden.)	a. C	
		b. C+aden.(5mM/L,23rd day)	
	D. ACDA(20mM/L aden.)	a. D	
		b. D+aden.(5mM/L,23rd day)	

Series D

Blood Number	Portions (Preservative conc.)	Division of Portions (additive,conc.,day)	Subdivision (additive, conc., day)
1	A. ACD	a. A	
		b. Hemolysate of A	
2	A. ACD	a. A	
		b. Hemolysate of A	(i) b
			(ii) b+aden.(20mM/L,10th day)
3	A. ACD	a. A	
		b. Hemolysate of A	(i) b
			(ii) b+aden.(20mM/L, 8th day)
4	A. ACD	a. A	
		b. Hemolysate of A	(i) b
			(ii) b+aden.(20mM/L,10th day)
			(iii) b+R-5-P(" ")
5	A. ACD	a. A	
		b. Hemolysate of A	(i) b
			(ii) b+aden.(20mM/L, 5th day)
			(iii) b+R-5-P(" ")
			(iv) b+ATP (" ")
			(v) b+ions.(" ")

TABLE 2

MATERIALS USED TO DETERMINE HEMOLYSATE PHOSPHOFRUCTOKINASE ACTIVITY

Materials*	Conc., mM/L.
Potassium fluoride	17
Tris(hydroxymethyl)-aminomethane buffer, pH 9.0	56
Sodium arsentate, pH 7.4	3
Potassium phosphate, pH 7.4	8
Cysteine	3
Hemolysate : 2.0 ml	
Aldolase : 50 ug in 0.1 ml of 0.53 M glycine	
Magnesium chloride	1.50
Diphosphopyridine nucleotide**	0.16
Potassium fructose-6-phosphate	1.40
Potassium adenosine triphosphate	0.40
Potassium fructose-1,6-phosphate	1.40
Sodium pyruvate	1.50

* Added in the order listed to each of a pair of cuvettes containing a final volume of 3.35 ml at pH 8.5 .

** Diphosphopyridine nucleotide was omitted from the blank cuvette.

TABLE 3

MATERIALS USED TO DETERMINE CRYSTALLINE ENZYME PURITY

Materials*	Test Systems I	II	III
Potassium fluoride	+	+	+
Tris(hydroxymethyl)-aminomethane	+	+	+
Sodium Arsenate	+	+	+
Potassium phosphate	+	+	+
Cysteine	+	+	+
Magnesium chloride	+	+	+
Aldolase	+	+	-
Potassium adenosine triphosphate	+	+	+
Diphosphopyridine nucleotide**	+	+	+
Potassium fructose-6-phosphate	+	+	+
Glyceraldehyde-3-phosphate dehydrogenase	+	-	-
Potassium fructose-1,6-phosphate	+	+	-
Glyceraldehyde-3-phosphate dehydrogenase	-	+	+
Potassium fructose-1,6-phosphate	-	-	+
Aldolase	-	-	+

* The materials in each of the test systems were added in the order listed to each of a pair of cuvettes containing a final volume of 3.35 ml. The concentrations of the materials were as listed in Table 2.

** Diphosphopyridine nucleotide was omitted from the blank cuvette.

TABLE 4

PHYSICAL TESTS APPLIED TO BLOODS

STORED IN VARIOUS MEDIA

Sample Number	Osmotic Fragility		Mechanical Fragility		M.C.H.C.	
	Fresh Blood	Stored 28 days	Fresh Blood	Stored 28 days	Fresh Blood	Stored 28 days
	%	%	%	%	%	%
B2-C-ACG	4	45	19	35	spontaneous	hemolysis
B4-A-ACD	5	28	20	25	33	35
B4-B-ACDA	5	7	20	21	33	33
B5-A-ACD	20	36	27	46	32	35
B5-B-AC	20	36	27	48	32	36
B5-C-ACDA	20	26	27	33	32	32
B5-D-ACA	20	25	27	36	32	32
B6-A-ACD	2	16	13	20	33	36
B6-B-AC	2	23	13	26	33	36
B6-C-ACDA	2	5	13	14	33	33
B6-C-ACA	2	3	13	15	33	33
C2-A-ACD					33	36
C2-B-ACDA					33	33

TABLE 5

A COMPARISON OF PHOSPHOFRUCTOKINASE ACTIVITY IN HEMOLYSATES PREPARED FROM WASHED AND UNWASHED ERYTHROCYTES OF THE SAME BLOOD SAMPLES

Number of Days Blood in Storage	PFK Activity(umoles DPN reduced/gm.Hb/min.) Washed	Unwashed
0	2.41	2.43
2	2.05	2.10
7	1.34	1.32
14	1.05	1.08
21	0.75	0.72
28	0.44	0.45

TABLE 6

A COMPARISON OF PHOSPHOFRUCTOKINASE ACTIVITY IN HEMOLYSATES PREPARED FROM DUPLICATE SAMPLES OF ERYTHROCYTES FROM VARIOUS STORED BLOODS

Blood Number	PFK Activity(umoles DPN reduced/gm.Hb/min.) Sample 1	Sample 2
A-3	2.71	2.68
A-4	2.00	1.97
B-5	1.53	1.56
B-6	1.24	1.23
C-1	0.05	0.05

Fig.I

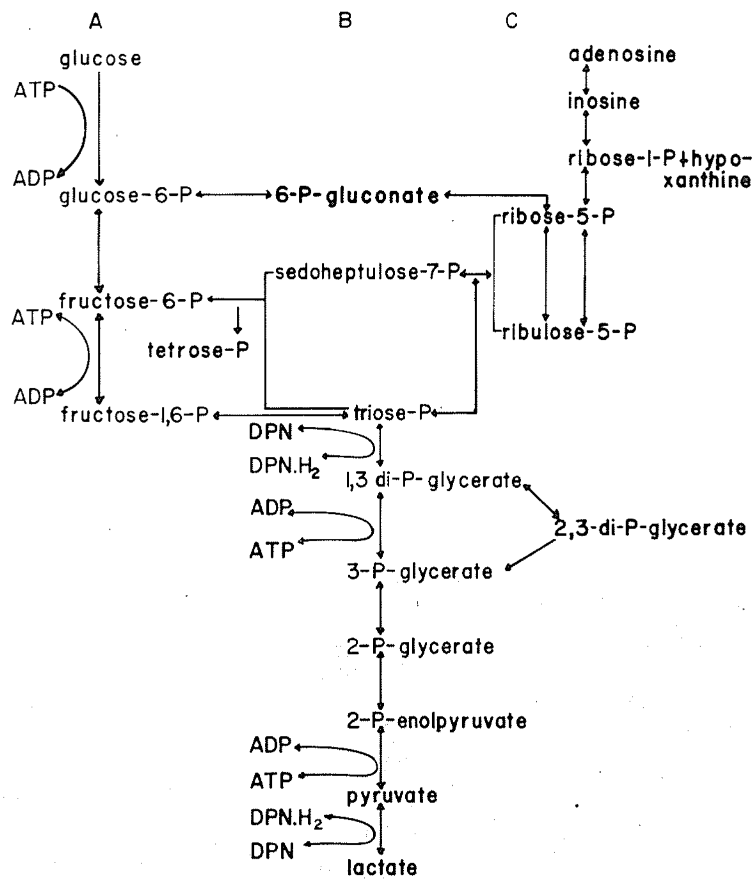


Figure 1. GLYCOLYSIS AND ASSOCIATED SCHEMES FOR THE METABOLISM OF GLUCOSE AND RIBOSE IN LIVING TISSUE:

A. Glycolysis; B. Hexosemonophosphate shunt; C. Pentose cycle adenosine metabolic pathway in the erythrocyte.

Fig. I

A B C

glucose
ATP
ADP
glucose-6-P ⟷ 6-P-gluconate
fructose-6-P
tetrose-P
ATP
ADP
fructose-1,6-P ⟷ triose-P
sedoheptulose-7-P
adenosine
inosine
ribose-1-P + hypoxanthine
ribose-5-P
ribulose-5-P
DPN
DPN.H_2
1,3 di-P-glycerate
2,3-di-P-glycerate
ADP
ATP
3-P-glycerate
2-P-glycerate
2-P-enolpyruvate
ADP
ATP
pyruvate
DPN.H_2
DPN
lactate

Figure 2. ENZYMES OF GLYCOLYSIS USED IN MEASURING PHOSPHOFRUCTOKINASE ACTIVITY IN HEMOLYSATE: I, phosphofructokinase; II, aldolase; III, triosephosphate isomerase; IV, d-glyceraldehyde-3-phosphate dehydrogenase. The abbreviations are as in the "GLOSSARY OF TERMS AND ABBREVIATIONS" and as follows: DHAP, dihydroxy acetone phosphate; 3-PG, 3-phosphoglycerate.

Fig. 2

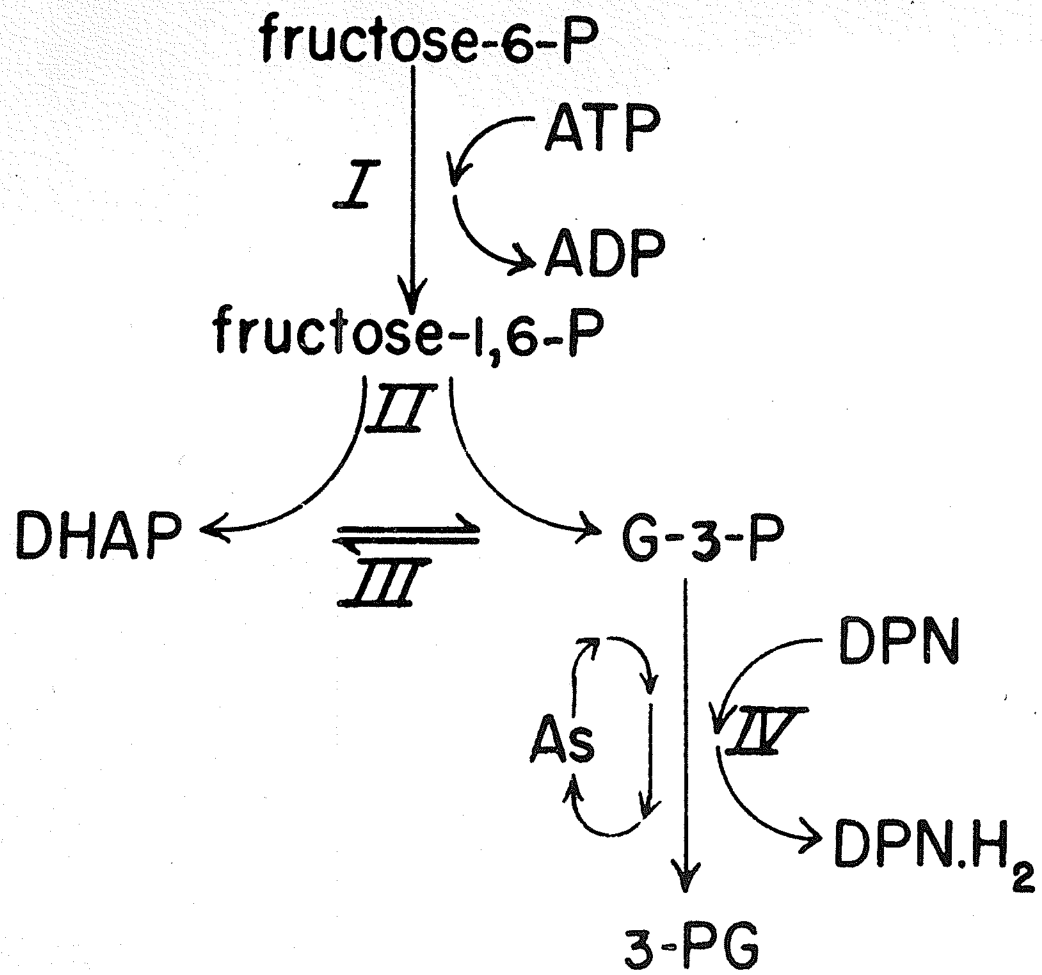


Figure 3. A TYPICAL PHOSPHOFRUCTOKINASE AND INDIGENOUS ENZYME ASSAY. Changes in optical density due to DPN reduction and DPN.H_2 oxidation. Curve 1, hemolysate with aldolase added; curve 2, hemolysate, no aldolase added. A, activity of indigenous enzymes; B, PFK activity; C, same as A, with aldolase added. Conditions and materials are given in Table 2 and in the text.

Figure 4. COMPARISONS AMONG A TYPICAL ASSAY AND SYSTEMS WITH ADDITIONAL SUBSTRATE AND WITHOUT ADDED SUBSTRATE. Changes in optical density due to DPN reduction and DPN.H_2 oxidation. Curve 1, usual PFK assay; curve 2, additional F-6-P added to assay system; curve 3, added F-6-P ommitted in the assay system B and C as in Fig. 3. Conditions and materials are given in Table 2 and the text.

Figure 5. COMPARISONS AMONG A TYPICAL ASSAY AND SYSTEMS WITHOUT ADDED ARSENATE OR PHOSPHATE. Changes in optical density due to DPN reduction and DPN.H_2 oxidation. Curve 1, usual PFK assay; curve 2, added phosphate ommitted in the assay system; curve 3, added arsenate ommitted in the assay system. B and C as in Fig. 3. Conditions and materials are given in Table 2 and in the text.

Figure 6. COMPARISONS AMONG ASSAYS OF VARIOUS DILUTIONS OF THE SAME HEMOLYSATE. Changes in optical density due to DPN reduction and DPN.H_2 oxidation. B and C as in Fig. 3. Conditions and materials are given in Table 2 and in the text.

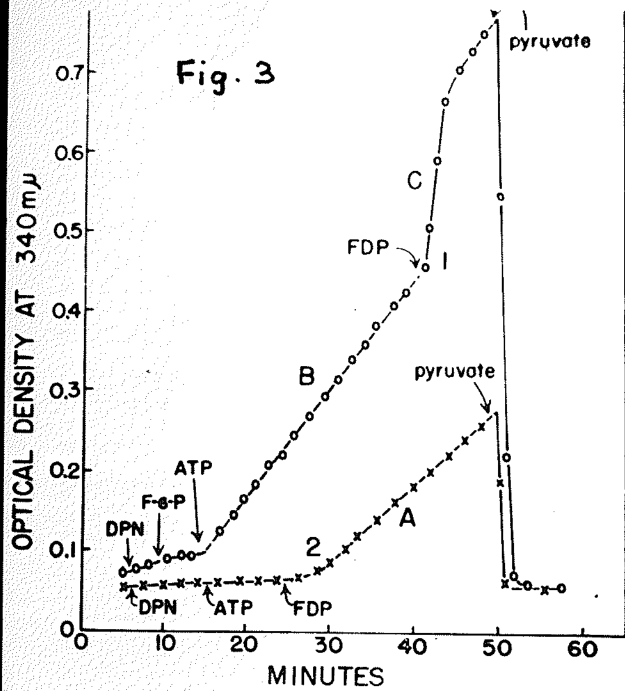


Fig. 3
OPTICAL DENSITY AT 340mμ
MINUTES
pyruvate
C
FDP
1
B
pyruvate
ATP
F-6-P
DPN
2
A
DPN
ATP
FDP

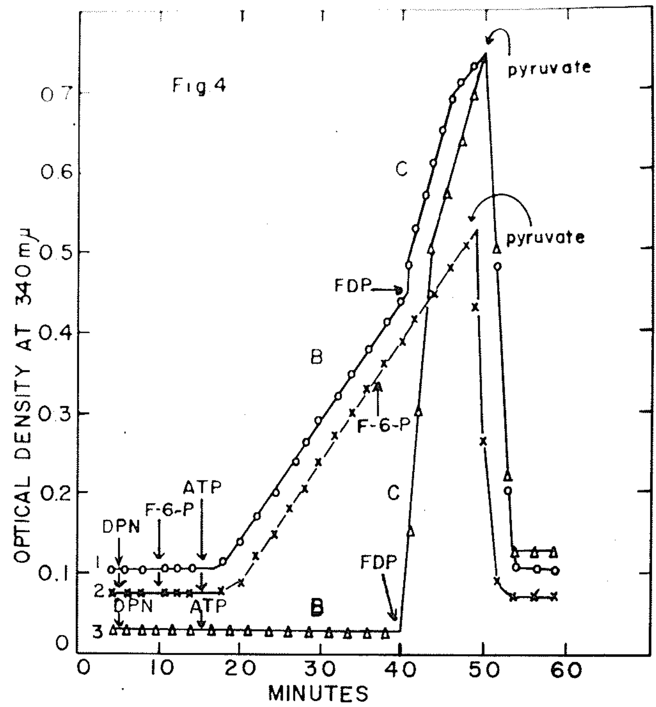


Fig 4
OPTICAL DENSITY AT 340mμ
MINUTES
pyruvate
C
pyruvate
FDP
B
F-6-P
ATP
F-6-P
DPN
C
FDP
DPN
ATP
B
1
2
3

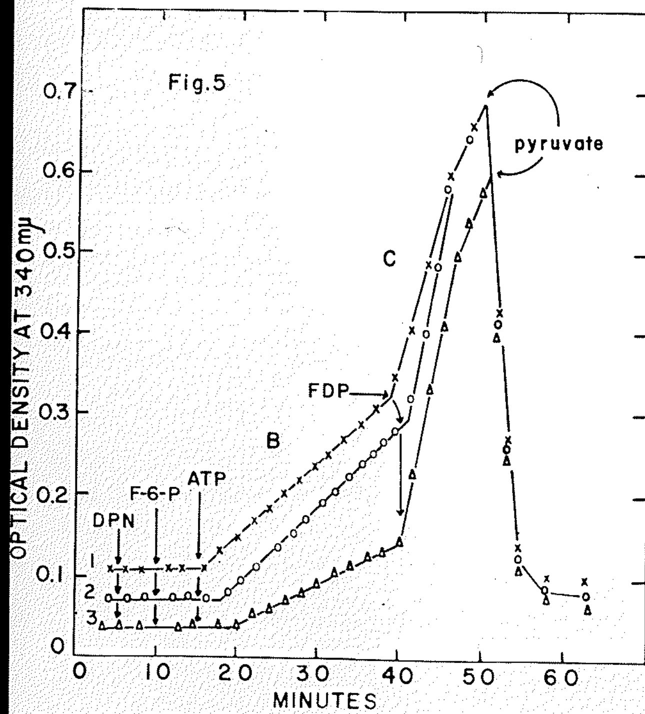


Fig.5
OPTICAL DENSITY AT 340mμ
MINUTES
pyruvate
C
FDP
B
ATP
F-6-P
DPN
1
2
3

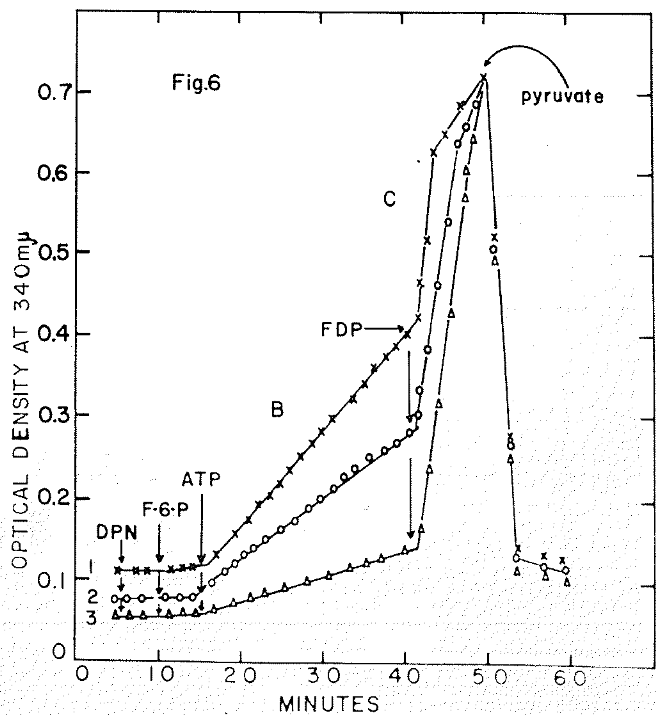


Fig.6
OPTICAL DENSITY AT 340mμ
MINUTES
pyruvate
C
FDP
B
ATP
F-6-P
DPN
1
2
3

Figure 7. A COMPARISON BETWEEN A TYPICAL INDIGENOUS ENZYME ASSAY AND A SYSTEM IN WHICH ADDED GLUTATHIONE REPLACES ADDED CYSTEINE. Changes in optical density due to DPN reaction and DPN.H_2 oxidation. Curve 1, usual indigenous enzyme assay; curve 2, indigenous assay with added glutathione in the system replacing added cysteine. Conditions and materials are those in Table 2 and the text.

Figure 8. A COMPARISON BETWEEN ASSAYS OF HEMOLYSATES PREPARED FROM "ACD-BLOOD" AND "ACDA-BLOODS". Changes in optical density due to DPN reduction and DPN.H_2 oxidation. Curve 1, hemolysate of "ACD-blood"; curve 2, hemolysate of "ACDA-blood"; curve 3, indigenous enzyme assay in hemolysate prepared from "ACDA-blood". A, B, and C as in Fig. 3. Conditions and materials are those in Table 2 and the text.

Figure 9. TESTS FOR ENZYME PURITY. Changes in optical density due to DPN reduction. Conditions and materials are those in Table 3 and the text.

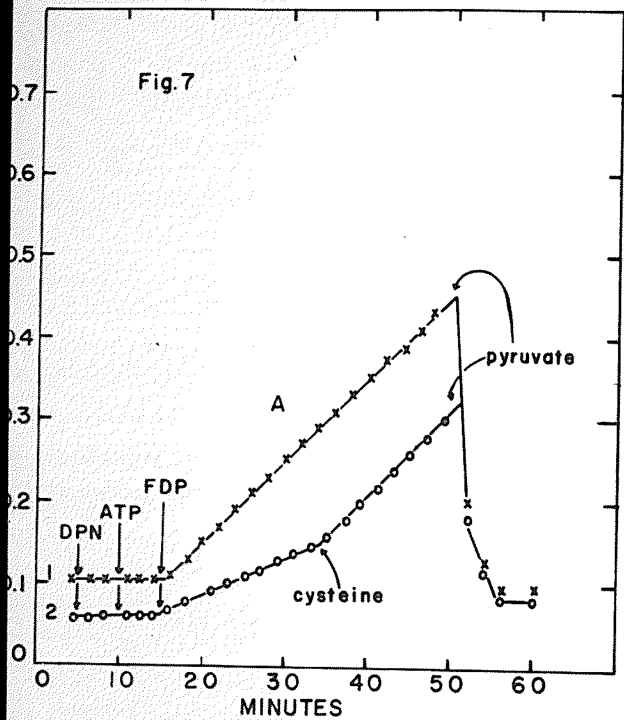


Fig.7
DPN
ATP
FDP
A
pyruvate
cysteine
MINUTES

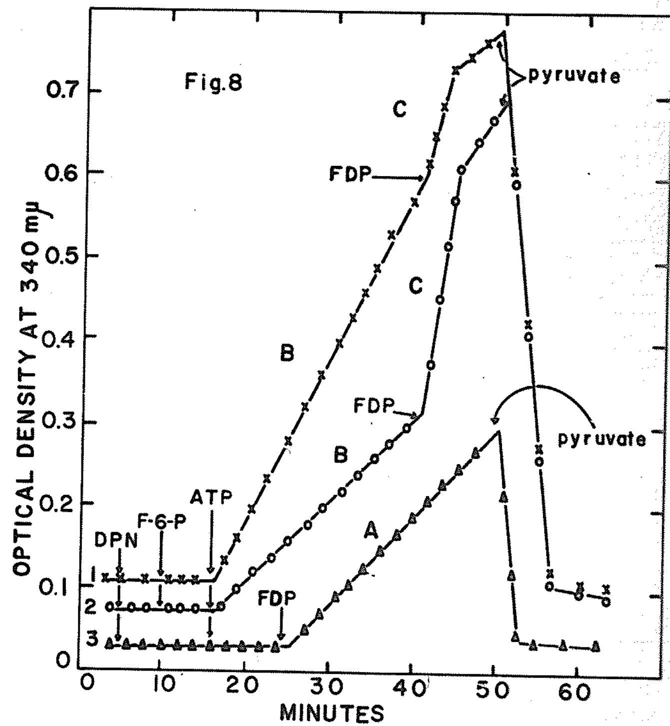


Fig.8
pyruvate
C
FDP
B
C
FDP
B
A
pyruvate
DPN
F-6-P
ATP
FDP
OPTICAL DENSITY AT 340 mμ
MINUTES

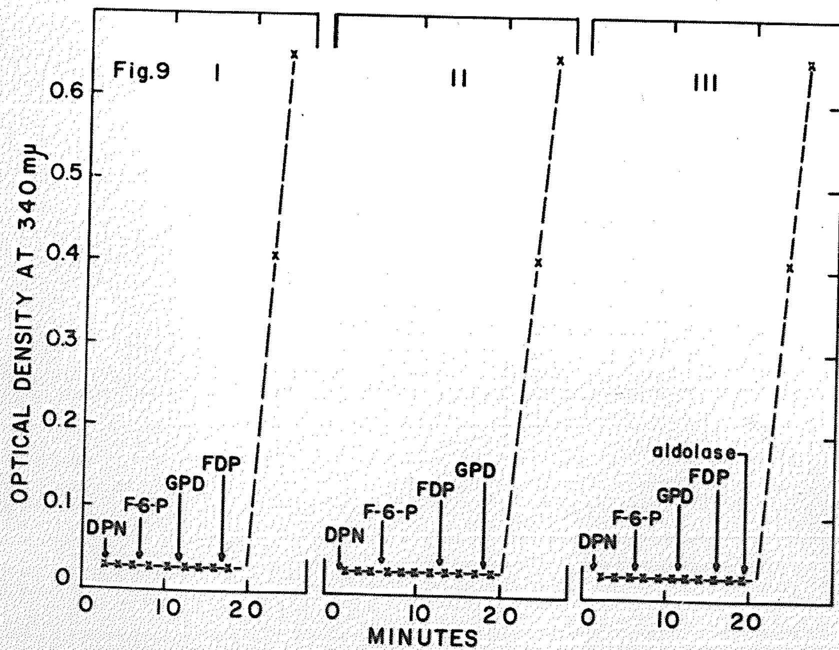


Fig.9
I
II
III
OPTICAL DENSITY AT 340 mμ
DPN
F-6-P
GPD
FDP
DPN
F-6-P
FDP
GPD
aldolase
DPN
F-6-P
GPD
FDP
MINUTES

Figure 10. EFFECT OF STORING BLOOD IN ACD ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES A, 1, 2, and 4: A, indigenous enzyme activity; B, PFK activity; C, same as A with added aldolase.

Figure 11. EFFECT OF STORING BLOOD IN ACD ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES B: A, indigenous enzyme activity; B, PFK activity; C, same as A, with aldolase added.

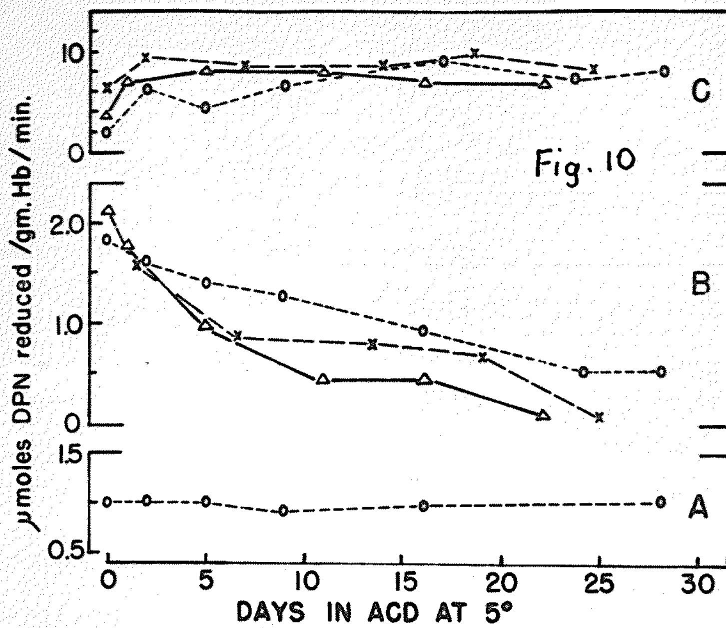


Fig. 10
μmoles DPN reduced /gm.Hb/min.
C
B
A
DAYS IN ACD AT 5°

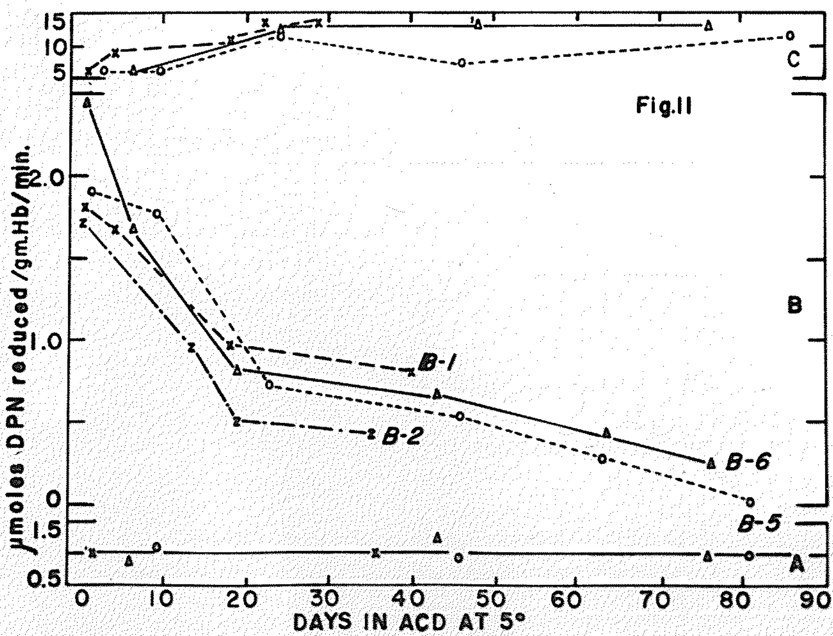


Fig.11
μmoles DPN reduced /gm.Hb/min.
C
B
B-1
B-2
B-6
B-5
A
DAYS IN ACD AT 5°

Figure 12. EFFECT OF STORING BLOOD IN ACD ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES C:

A, indigenous enzyme activity; B, PFK activity;

C, same as A, with added aldolase.

Figure 13. EFFECT OF STORING BLOOD IN ACD AND THE EFFECT OF STORING HEMOLYSATE PREPARED FROM THE SAME "ACD-BLOODS" ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES D:

I Blood stored in ACD.

II Hemolysates prepared from the same bloods.

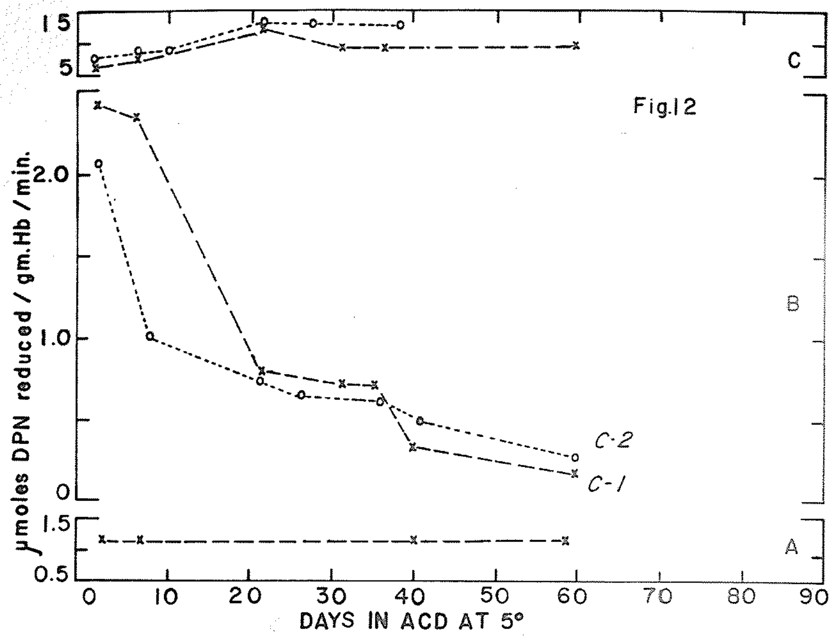


Fig.12
µmoles DPN reduced / gm. Hb / min.
C
B
A
C-2
C-1
DAYS IN ACD AT 5°

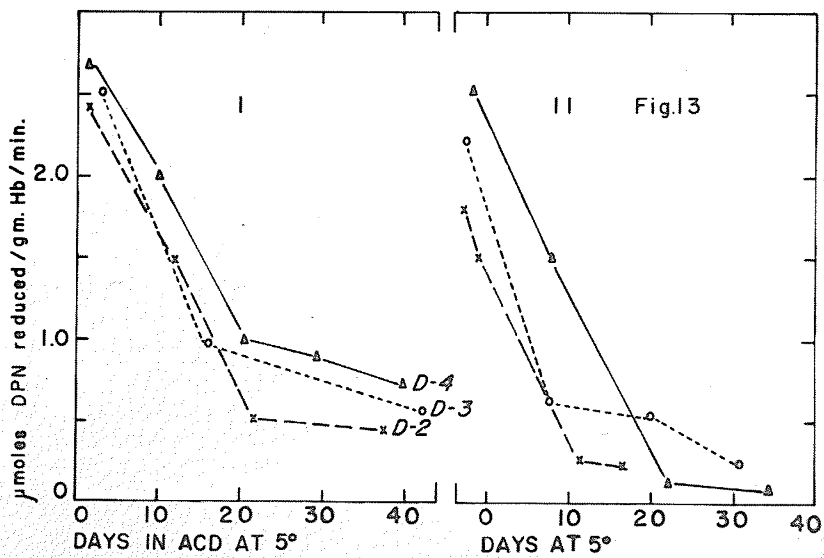


Fig.13
I
II
µmoles DPN reduced / gm. Hb / min.
D-4
D-3
D-2
DAYS IN ACD AT 5°
DAYS AT 5°

Figure 14. EFFECT OF STORING PORTIONS OF THE SAME BLOOD IN ACD, AC, ACDA, AND ACA ON THE RATE OF DPN REDUCTION BY HEMOLYSATES SERIES B-5:

PFK activity is shown.

Figure 15. EFFECT OF STORING PORTIONS OF THE SAME BLOOD IN ACD, AC, ACDA, AND ACA ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES: B-6:

PFK activity is shown.

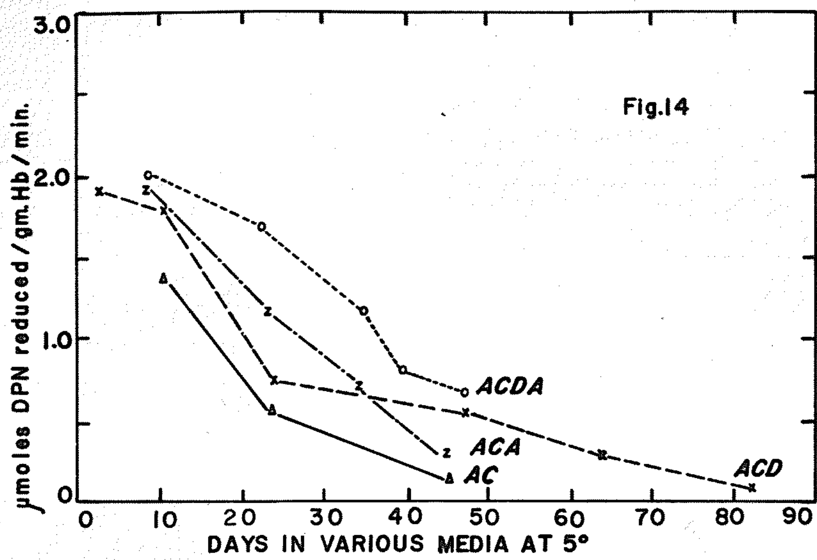


Fig.14
μmoles DPN reduced / gm.Hb / min.
3.0
2.0
1.0
0
ACDA
ACA
AC
ACD
0 10 20 30 40 50 60 70 80 90
DAYS IN VARIOUS MEDIA AT 5°

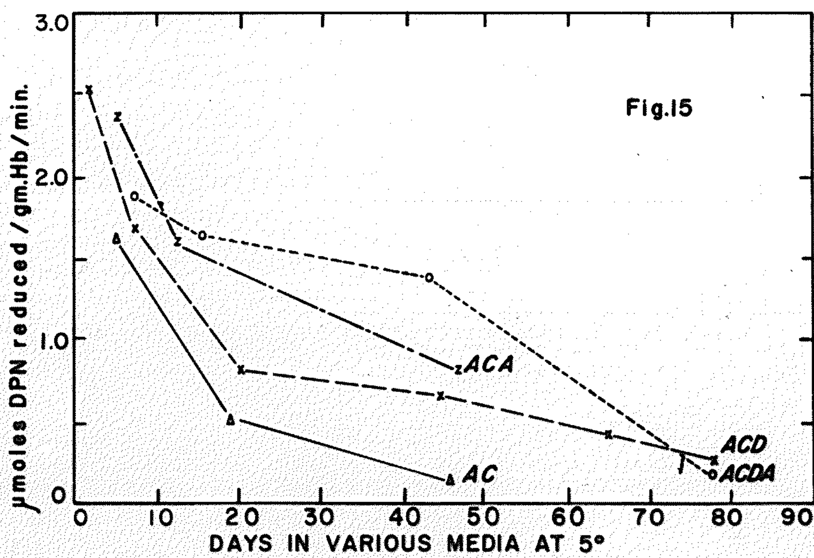


Fig.15
μmoles DPN reduced / gm.Hb / min.
3.0
2.0
1.0
0
ACA
ACD
ACDA
AC
0 10 20 30 40 50 60 70 80 90
DAYS IN VARIOUS MEDIA AT 5°

Figure 16. EFFECT OF STORING PORTIONS OF THE SAME BLOOD IN ACD, AC, ACDA, AND ACA AND ADDING ADENOSINE TO ALIQUOTS OF THESE PORTIONS DURING STORAGE ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES C-1:

PFK activity is shown.

Figure 17. EFFECT OF STORING PORTIONS OF THE SAME BLOOD IN ACD, AND ACDA AND ADDING ADENOSINE TO ALIQUOTS OF THESE PORTIONS ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES C-2:

PFK activity is shown.

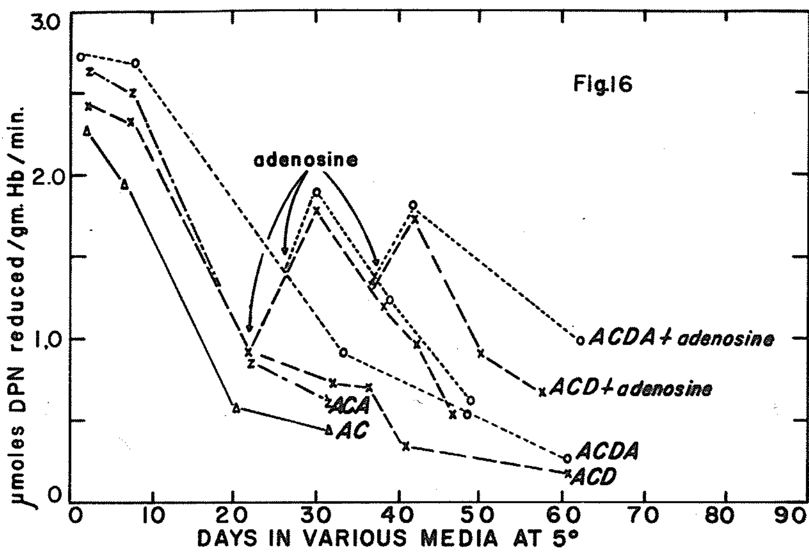


Fig.16
adenosine
ACDA + adenosine
ACD + adenosine
ACA
AC
ACDA
ACD
μmoles DPN reduced/gm. Hb/min.
DAYS IN VARIOUS MEDIA AT 5°

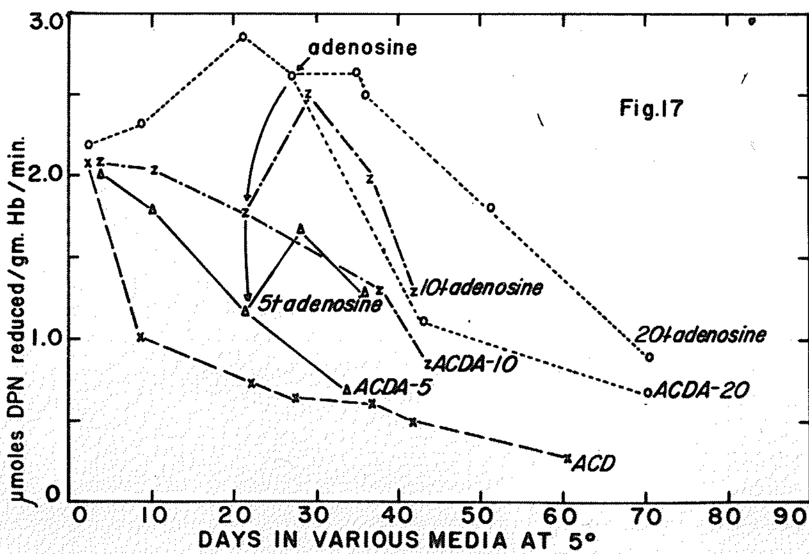


Fig.17
adenosine
10+adenosine
5+adenosine
20+adenosine
ACDA-10
ACDA-5
ACDA-20
ACD
μmoles DPN reduced/gm. Hb/min.
DAYS IN VARIOUS MEDIA AT 5°

Figure 18. EFFECT OF STORING HEMOLYSATES PREPARED FROM "ACD-BLOOD" WITH AND WITHOUT ADDED ADENOSINE ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES D-2 and 3: PFK activity is shown.

Figure 19. EFFECT OF STORING HEMOLYSATES PREPARED FROM "ACD-BLOOD" WITH AND WITHOUT VARIOUS PRESERVATIVES ON THE RATE OF DPN REDUCTION BY HEMOLYSATES: SERIES D-4 and 5: PFK activity is shown

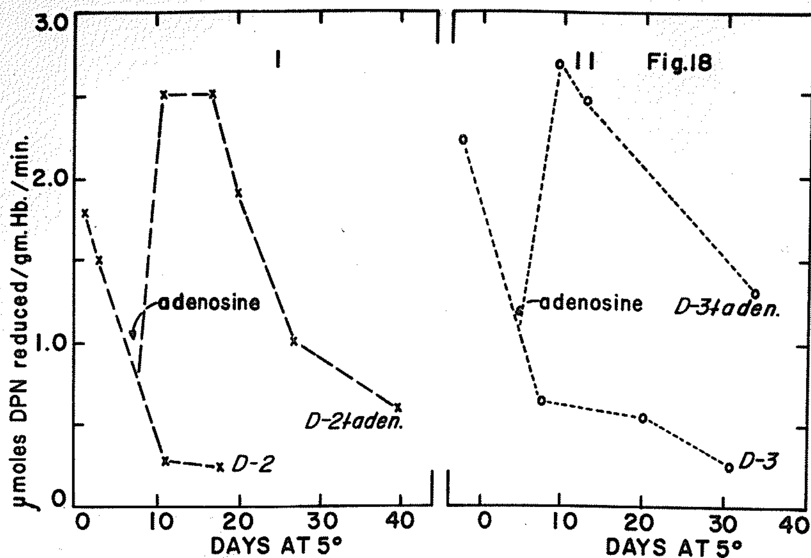


Fig.18
I
II
μmoles DPN reduced/gm.Hb./min.
adenosine
D-2+aden.
D-2
adenosine
D-3+aden.
D-3
DAYS AT 5°
DAYS AT 5°

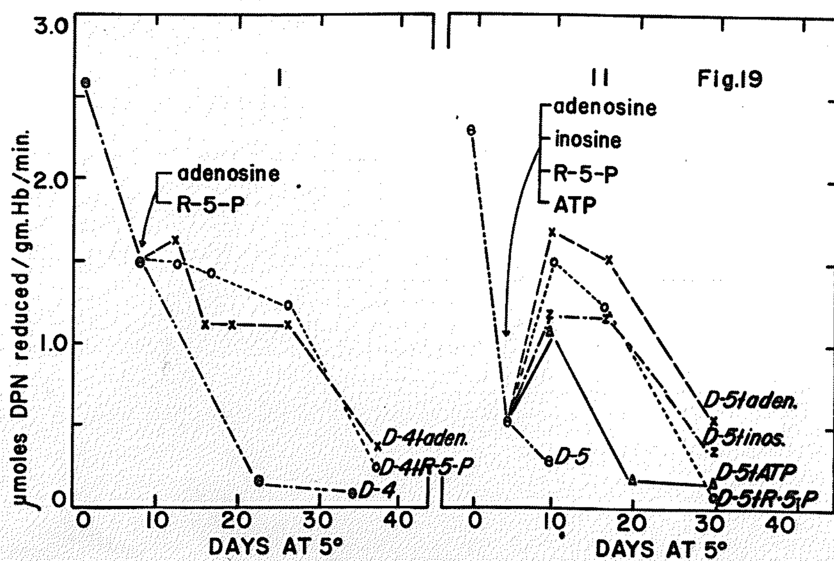


Fig.19
I
II
μmoles DPN reduced/gm.Hb./min.
adenosine
R-5-P
D-4+aden.
D-4+R-5-P
D-4
adenosine
inosine
R-5-P
ATP
D-5+aden.
D-5+inos.
D-5+ATP
D-5+R-5-P
D-5
DAYS AT 5°
DAYS AT 5°

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